Office Action mailed September 11, 2001, and respectfully submits that the claims are now in condition for allowance.

Amendment of the Specification

The specification has been amended in order to more clearly set forth what is intended as Applicant's invention, or to correct a typographical or clerical error. The chemical structures depicted on pages 74-77 and 79-80 have been amended and redrawn to overcome a lack of clarity. Applicant respectfully submits that no new matter is added with these amendments and additions.

Addition of Claims

Claims 57-81 have been newly added and Applicant submits that these additions do not present new matter. Specifically, support for newly added claims 57 and 58 can be found *inter alia* in original claim 1 and throughout the specification. With respect to the ligand, the term "associate with" is defined in the paragraph bridging pages 23 and 24 of the specification.

Support for claim 59 can be found on page 24 lines 8-19 where it is recited that a biological component may be any detectable compound or portion of a compound that (i) is found in a cell; (ii) participates in one or more biological reactions; and/or (iii) is produced by one or more biological reactions. Page 25 lines 6-7 reads: "Typically, but not necessarily the detection target will be a product of or a participant in the reaction being studied". Additional support can also be found on page 6 lines 17-22 of the specification, where it is recited that, in certain preferred embodiments, the invention provides a screening method for identifying compounds capable of affecting a biological or chemical process by detecting levels of component in the biological or chemical process (*i.e.*, a component that participates in the chemical or biological process) or resulting from the biological or chemical process (*i.e.*, the component is a product of the chemical or biological process).

Support for claims 60-66 can be found, for example, in the paragraph starting on page 5 line 17 and ending on page 6 line 5, in the "Detection" section on pages 35-38, and throughout

the Examples, where it is recited that the biological component(s) is (are) detected by means of its (their) interaction with a binding partner ligand, that the binding may be specific, and that in certain embodiments, the binding partner ligand is an antibody and may include a peroxidase, for example horseradish peroxidase (HRP). Claim 64 includes the limitation that detection of the bound second ligand may be performed while the bound ligand is inside the cell (*e.g.*, without requiring cell lysis). Support for such language can be found throughout the Examples in the teaching that the bound second ligand (*e.g.*, antibody conjugated to horseradish peroxidase) is detected in intact cells.

Claim 67 recites elements pertaining to detection methods (support for which can be found on page 37 lines 3-12 and on page 38 lines 13-23).

Claims 68 and 69 include the use of an assay solution containing a reagent for detecting the presence or amount of a biological component (detection target). Support for such language can be found on page 6 lines 17-22. Further support is provided in Figure 5 and Example 4 (pages 56-58) where it is described that the reagent (*e.g.*, trapoxin A) may exert an effect on the intracellular biological or chemical process of interest (*e.g.*, protein phosphorylation). Additional support can be found on page 31 lines 1-12, in Figures 1-4, 8, 11c-d and in Examples 1-3, 6-7 (pages 48-63), where it is recited that the reagent (*e.g.*, BrdU) may be incorporated in the biological component resulting from the intracellular biological or chemical process of interest (*e.g.*, DNA synthesis).

Claim 70 includes covalent modifications of intracellular components as markers for detecting the effects of test compounds on intracellular biological and chemical processes. Support for this language can be found throughout the specification, particularly on page 31 lines 23-24 and page 32 lines 1-15, which recite the detection of covalent modifications (*e.g.*, phosphorylation or acetylation) to identify test compounds capable of affecting various cellular functions.

Claims 71 and 72 recite biosynthetic events as examples of covalent modification of intracellular components, support for which can be found *inter alia* in original claim 9.

Support for newly added claims 73 and 74 can be found on page 56 lines 23-23 and page 96 lines 11-13, in which it is recited that in certain embodiments that compounds can be screened for their effect on post-translational events. It is well recognized in the art that the cellular processes listed on page 42 lines 9-13 are post-translational events.

Claim 75 specifies that the ligand interacts with the post-translationally modified intracellular component (*i.e.*, a biological component product of a post-translational biological process). Support for this language can be found, for example, on page 24 lines 8-19, page 25 lines 6-7 and page 6 lines 17-22 as detailed above for claim 59. Additional support can be found, for example, in Example 4 (pages 56-58 of the specification) in the teachings that an antibody (*i.e.*, ligand) can be used to detect the presence of phosphorylated nucleolin or histone H3 in the cells.

Claims 76 and 77 find support *inter alia* in original claims 6 and 7, respectively.

Claims 78-80 recite elements pertaining to the assay format. Specifically, claim 78, which includes elements pertaining to the volume of the reaction vessels of the assay format, finds support *inter alia* in original claim 14 and on page 6 lines 6-16; Claim 79, which is drawn to elements regarding the spacing of the reaction vessels of the inventive assay, finds support on page 5 lines 3-9 and page 29 lines 1-5; Finally, claim 80, which provides recitation of elements relative to the reaction vessel density of the assay used in the invention, finds support, for example, in Example 1 found on pages 48-51, in the teachings that a 384-well plate can be used to practice the invention. A person of ordinary skill in the art would appreciate that the dimension of standard 384-well plates used in the art is approximately 128×86 mm^2.

Claim 81 was drawn to a method of screening test compounds for establishing their functional fingerprint. Support for such addition can be found on page 41 line 22 through page 42 line 14, as well as in Example 12 on page 97-98 and Figure 13.

Applicant respectfully submits that addition of the claims, as described above and detailed herein, does not present new matter, and Applicant thus respectfully requests entry of these additions, and consideration of these additions in the following remarks.

Rejection of claims 41-51 and 53 under 35 U.S.C. § 112, first paragraph

Claims 41-51 and 53 were rejected under 35 U.S.C. § 112, first paragraph. The Examiner has argued that the present specification does not reasonably provide enablement for a method for screening ***all*** libraries of ***all*** test compounds with effects on ***all*** biological processes in ***all*** cells, comprising the steps of... providing a plurality of ***all*** cells; providing ***all*** libraries of ***all*** test compounds to be assayed for ***all*** biological processes in ***all*** cells, wherein ***all*** biological processes are characterized in that production of ***all*** intracellular products indicate activities of ***all*** of the aforementioned processes... wherein each reaction vessel contains ***a/all*** subset(s) of ***all*** libraries of ***all*** test compounds, contacting ***all*** test compounds with ***all*** cells for ***all*** periods of time and under ***all*** conditions sufficient for ***all*** test compounds to exert an effect on ***all*** biological processes so that ***all*** levels of ***all*** intracellular products are affected, introducing into each reaction vessel ***all*** ligands that bind specifically ***all*** intracellular products in ***all*** biological processes so that ***all*** ligands bind to ***all*** products..., etc. However, the Examiner has conceded that the claims are enabling for "screening strategies associated with the following different classes of chemical compounds as affecting aspects of the cell cycle, as indicated by these examples from the instant specification:" citing Examples 6, 7, 9, 10 and 11.

Applicant respectfully disagrees that the specification does not reasonably provide enablement commensurate with the scope of the claimed invention, and instead submits that the specification provides sufficient guidance for one of ordinary skill in the art to make and use the invention without undue experimentation. However, in order to expedite prosecution, Applicants have canceled claims 41-51 and 53, and have added claims 57-81. The rejection under 35 U.S.C. § 112, first paragraph will be addressed as if it were applied to newly added claims 57-81.

The standard for enablement is based on the determination of whether the disclosure contains sufficient information regarding the subject matter of the claim as to enable one skilled in the art to make and use the claimed invention. As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied (*In re*

Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); MPEP 2164.01(b)). Applicant is not required to disclose every operable species, but only representative examples, with enough teaching and guidance so as to enable a person of ordinary skill in the art to practice the invention without undue experimentation.

Applicant respectfully submits that the specification ***does*** provide sufficient guidance and ***does*** teach one of ordinary skill in the art how to screen test compounds capable of exerting an effect on intracellular biological or chemical processes, ***without undue experimentation.*** Specifically, Applicant claims a method for screening compounds, said method comprising:

a. providing a plurality of reaction vessels;

Applicant asserts that there is no undue experimentation in providing a plurality of reaction vessels. Specifically, in describing a method of practicing the screening method of the invention, Applicant teaches the use of plates containing 384 (Nalge Nunc International, Naperville, IL; Greiner America, Lake Mary, FL; Corning Costar, Corning, NY) or 1536 (Greiner America, Lake Mary, FL) wells, which were commercially available at the time the invention was made (see p. 29 lines 6-19). Applicant further discloses that denser plates, such as the 6144 well plates described by You *et al.* (*Chem. Biol.* 4:969-975. 1997; U.S.S.N.# 09/184,449 entitled "Casting of Nanowell Plates") are particularly preferred for practicing the invention, and that an ideal assay for high throughput screening would be compatible with any or all of the above-mentioned array formats. The reaction vessel assay formats needed to practice the invention were well-known in the art at the time the invention was made, and were readily available to the person of ordinary skill in the art who would want to make or use the invention. Thus Applicant maintains that the step of providing a plurality of reaction vessels cannot rise to the level of undue experimentation.

b. providing a plurality of cells;

Applicant respectfully submits that there is no undue experimentation in providing a plurality of cells. Specifically, Applicant teaches the use of a variety of cell types that can be

grown or delivered at the bottom of the plurality of reaction vessels described in the present invention, and probed for a specific biological component (*e.g.*, detection target) produced by an intracellular biological or chemical process of interest. Any cells can be used, including for example bacterial cells, yeast cells, plant cells, insect cells or animal cells (see page 30 lines 1-4). As stated on page 48 lines 9-11, the invention does not require the use of (but *can* use) engineered cell lines, which means that a wide range of cells including both primary and transformed cells (*i.e.*, cells that were not derived from a cancer but were produced by laboratory treatment of normal cells) of any tissue type or genetic background can be used (see page 48 lines 10-11). As seen in the Examples and Figures provided, they can include Mv1Lu and 6F mink lung epithelial cells (Figures 2-4, 6a, 7 and 8a-b), A549 human lung carcinoma cells (Figures 5, 6b-c 11b and 11e), HeLa human cervical carcinoma cells (Figures 6c and 11c-d), and mouse embryonic stem cells (Figure 8c). While the examples in the specification teach the use of several types of cell-lines in the present invention, the scope of the invention is not limited to screening only those cell-lines described in the Examples. It is clear that the present specification teaches those skilled in the art how to use ***any*** cell(s) that can be grown, cultured and/or assayed according to Applicant's screening method and that the present specification fully enables the screening of any library of test compounds using ***any*** cell type without undue experimentation. It is readily appreciated by those of ordinary skill in the art, that practice of the screening method taught in the invention is not restricted to the aforementioned cell types. Any cell known and available in the art may be used. Therefore the step of providing a plurality of cells cannot require undue experimentation.

c. providing one or more test compounds;

Applicant asserts that there is no undue experimentation in providing one or more test compounds for practicing the invention. Those of ordinary skill in the art readily appreciate that the present invention provides a method of screening compounds and that it can be used to screen ***any*** collection of test compounds. Applicant teaches that the present invention may be practiced with test compounds obtained from natural or synthetic sources. General descriptions

and references of libraries of natural and synthetic compounds are found in the specification on pages 39-40. Applicant teaches that traditionally, the source of test compounds for use in biological assays has been extracts containing natural products. However the advent of combinatorial chemistry and split-pool synthesis now adds to this repertoire complex "natural-product-like" libraries, the products of laboratory syntheses, as a source of small molecules to be screened for novel compounds with biological activity. While the examples in the specification teach the use of several classes of compounds in the present invention, the scope of the invention is not limited to screening only those compounds described in the Examples. It is clear that the present specification teaches those skilled in the art how to screen ***any*** test compound(s) that can be obtained through synthetic methods or isolated from natural or biological sources. Furthermore, given the high level of knowledge and high skill in the art, those skilled in the art readily appreciate that any collection of test compounds available to them can be screened using the presently claimed invention. As such, there is no undue experimentation in ***providing*** one or more test compounds for screening because ***any*** compound can be tested and ***any*** collection of test compounds can be screened.

d. introducing at least a subset of said cells into each of said reaction vessels;

The methods needed to perform the step of introducing the cells in the reaction vessels were well-known in the art at the time the invention was made. Applicant asserts that there is no undue experimentation in introducing the cells in each reaction vessel.

e. introducing at least one said test compound into each of said reaction vessels;

Given the high level of skill in the art at the time the invention was made, the step of introducing the test compounds in each reaction vessel cannot rise to the level of undue experimentation, since the methods needed to perform this step were known and readily available to a person of ordinary skill in the art.

f. contacting said test compounds with said cells in each of said reaction vessels under conditions suitable for at least one of the test compounds to exert an effect on an intracellular biological or chemical process;

Given the high level of skill in the art at the time the invention was made, the step of contacting the test compounds with the cells in each reaction vessel cannot rise to the level of undue experimentation. Applicant has provided ample teaching in the specification, particularly in the Examples, of methods suitable to carry out this step. In addition, Applicant teaches experimental conditions suitable for test compounds, when contacted with various cells, to exert an effect on a variety of intracellular biological or chemical processes including, but not limited to, gene expression, DNA synthesis, protein acetylation, protein phosphorylation, cell growth and mitosis (see Examples 1-14 on pages 48-105). Applicant is not required to disclose every operable species, but only representative examples. Thus, the scope of the claimed invention is not limited to the recited examples, but encompasses any intracellular biological or chemical process known in the art, that can be probed using the present invention. One skilled in the art can use and apply the teachings of the invention to screen compounds to identify those compounds that affect intracellular biological or chemical processes other than those disclosed in the present invention. As such, there is no undue experimentation in contacting the test compounds with the cells as set forth in the claims.

g. contacting a ligand with said cells in each reaction vessel under conditions suitable for said ligand to associate intracellularly with at least one biological component whose presence or amount is indicative of said biological or chemical process;

Given the high level of skill in the art at the time the invention was made, the step of contacting the ligand with the cells in each reaction vessel cannot rise to the level of undue experimentation. Applicant has provided ample teaching in the specification, particularly in the Examples, of methods suitable to carry out this step. In addition, Applicant teaches throughout the Examples experimental conditions suitable for the ligand to associate with a target biological component (*e.g.*, detection target) intracellularly. The term "associate with" is defined in the

paragraph bridging pages 23 and 24. While the Examples in the specification teach the use of several types of ligands, the scope of the invention is not limited to using only those ligands as detecting agents. Applicant teaches the general characteristics of suitable ligands to be used in the present invention on pages 26 and 27 of the specification (see also the paragraph starting on page 35 line 19 and ending on page 36 line 16). Those of ordinary skill in the art would appreciate that a wide variety of ligands and established specific associations are known in the art. One practicing the invention would choose a chemical or biological process of interest to them from a number of different chemical or biological processes which could be assayed for, and would select a ligand suitable for detecting the biological target; therefore a practitioner will not be at a loss as to how to perform the assay. Applicant submits that the specification provides sufficient guidance and teaching to enable one of ordinary skill in the art to practice the invention using other ligands known in the art. Therefore the step of contacting the ligands is fully supported and enabled by the specification.

h. measuring the presence or amount of said ligand associated to said biological component.

Applicant respectfully submits that there is no undue experimentation in measuring the presence or amount of biological component-associated ligand. Specifically, Applicant teaches detection methods suitable for practicing the present invention in the specification on pages 37-38 and throughout the Examples.

Additionally, Applicant would like to point out that, although the specific application of the claimed method using different test compounds, cell-lines or ligands other than those described in the application might involve some experimentation, the enablement requirement is still satisfied since the amount of experimentation would not be undue. *In re Vaeck*, 20 USPQ 2d 1438 (Fed. Cir. 1991) ("That some experimentation may be required is not fatal; the issue is whether the amount of experimentation is 'undue'."). As the Federal Circuit explained in *Genetech v. Novo Nordisk*, "undue experimentation" refers to the failure to disclose "any specific

starting material or of any of the conditions under which a process can be carried out." *Genentech v. Novo Nordisk*, 42 USPQ2d 1001 (Fed. Cir. 1997). For example, as detailed above and as recited from the specification, Applicant has provided sufficient guidance to the worker of ordinary skill in the art to obtain a variety of test compounds from synthetic or natural sources, to obtain a variety of cells (whether they be primary and transformed cells, from any tissue type or genetic background), to obtain a suitable ligand as detecting agent, and to screen the test compounds for their ability to exert an effect on an intracellular biological or chemical process of interest, and thus has provided specific starting materials (*e.g.*, test compounds, cells and ligands) and conditions under which the test compounds ability to affect cellular processes can be assessed.

In summary, Applicant asserts that the newly added claims are fully supported and enabled by the specification. The specification provides sufficient guidance and teaching to enable one of ordinary skill in the art to practice the invention commensurate in scope with the claims. In view of the arguments presented above and entering of amendment of newly added claims 57-81, Applicant respectfully submits that the claims are indeed enabled, and thus respectfully requests that the Examiner reconsider and withdraw the rejection of claims 41-51 and 53 under 35 U.S.C. § 112, first paragraph.

Rejection of claims 41-42 and 53 under 35 U.S.C. § 112, second paragraph

The Examiner has rejected claims 41-42 and 53 as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Cancellation of these claims renders the rejection moot. Furthermore, newly added claims 57-81 do not include the language said to be indefinite.

Rejection of claims 1-2, 5-6, 9-10, 13-14, 18-19, and 22-23 under 35 U.S.C. § 103 over:

1) Gallop et al. (USP 5,525,734; "Gallop"), Manns (USP 4,948,442; "Manns"), page 29, lines 6-19 of the present application, and Craig ("Chapter 14, Screening Combinatorial

Libraries," A Practical Guide to Combinatorial Chemistry, Czarnik and DeWitt eds., Washington D.C.: American Chemical Society, 1997; "Craig");

2) Zambias et al. (USP 5,736,412; "Zambias"), Manns, page 29, lines 6-19 of the present application, and Craig; and

3) Godowski et al. (USP 6,025,145; "Godowski"), Manns, page 29, lines 6-19 of the present application, and Craig.

Claims 1-2, 5-6, 9-10, 13-14, 18-19, and 22-23 have been canceled and replaced with claims 41-56 pursuant to Applicant's June 8, 2001 Response. The rejections under 35 U.S.C. §103 were maintained in the Examiner's Final Office Action dated September 11, 2001. Claims 41-56 have been canceled and new claims 57-81 have been added, thereby rendering the rejection under 35 U.S.C. §103 moot. To provide a clear record, Applicant provides arguments below that this rejection should not be applied to new claims 57-81. Specifically, Applicant submits that claims 57-81 are not obvious over 1) Gallop, Manns, page 29 lines 6-19 of the present application and Craig; 2) Zambias, Manns, page 29 lines 6-19 of the present application and Craig; and 3) Godowski, Manns, page 29 lines 6-19 of the present application and Craig.

The Examiner states that Gallop teaches methods of synthesizing and screening pyrrolidine compound libraries on solid supports. The Examiner asserts that Gallop also teaches screening the library for biological or pharmaceutical activity. However, the Examiner has conceded that Gallop does not suggest or teach a method of screening compounds where the volume of each reaction vessel is less than or equal to approximately 200 microliters. In addition, Applicant submits that there is no indication or teaching in Gallop of the use of a cell-based assay for performing the biological assay. Furthermore, Gallop does not teach the ability of the compounds to exert an effect on intracellular processes as a basis for the biological screen, and that this effect may be detected via a ligand (or combination of ligands) that associates with the target biological component ***inside the cell***.

The Examiner asserts that Zambias teaches a method of synthesizing an (m x n) array of different chemical compounds wherein each of the compounds has at least one structural diversity element selected from a group of amines and ketones and wherein the scaffold structure is selected from a group consisting of aminimide, imidazolone, sulfonylaminimide and phosphonylaminimide (see abstract of Zambias). The Examiner states that Zambias teaches simultaneous screens for assaying large numbers of parallel compound samples for exploring biological activity. However, the Examiner has conceded that Zambias does not suggest or teach a method of screening compounds where the volume of each reaction vessel is less than or equal to approximately 200 microliters. In addition, Applicant submits that there is no indication or teaching in Gallop of the use of a cell-based assay for performing the biological assay. Furthermore, Gallop does not teach the ability of the compounds to exert an effect on intracellular processes as a basis for the biological screen, and that this effect may be detected via a ligand (or combination of ligands) that associates with the target biological component ***inside the cell***.

The Examiner asserts that Godowski teaches a kinase receptor activation (KIRA) assay for measuring autophosphorylation of a tyrosine kinase receptor of interest. Godowski's method involves contacting cells with a compound to assay the ability of the compound to affect activation of the auto-phosphorylation process. After the cells are exposed to the compound, the cells are lysed. The lysate is removed and transferred to a second solid phase containing a capture agent which binds specifically to the tyrosine kinase receptor. The second solid phase is washed to remove unbound cell lysate, leaving the captured receptor. The captured tyrosine kinase receptor is then contacted with anti-phosphotyrosine antibodies (*e.g.*, detecting ligand) which are specific to phosphorylated residues and the levels of the antibodies are measured. However, the Examiner has conceded that Godowski does not suggest or teach a method of screening compounds where the volume of each reaction vessel is less than or equal to approximately 200 microliters. Furthermore, Applicant submits that Godowski's assay differs from Applicant's invention in that it does not teach the introduction of a ligand for associating

with the biological components of interest ***intracellularly***. Godowski's invention relates to a high throughput format using immobilized cell lysates at the bottom of assay plates as the source of antigens for detection in an enzyme-linked immunosorbent assay (ELISA). In other words, Godowski teaches an assay whereby the cells are solubilized and the receptors immobilized on a second solid support prior to exposing the receptors to a ligand for detection. Applicant's screening assay relies on the introduction of the detecting ligand inside the cell where it associates with the biological component whose presence or amount is indicative of the biological or chemical process of interest. In fact, Applicant's invention has the advantage over Godowski's of preserving cellular architecture, as well as not requiring cell lysis and subsequent lysate transfer, which are potentially rate-limiting and variable steps when dealing with large numbers of samples.

Manns teaches a standard 96 well micro-titer test plate and methods for producing the plates where the plates contain an incubation tray, a filter and a harvester tray having mating ridges and grooves to prevent cross-talk between the wells along the filter (see Abstract). Manns does not teach cell-based assays. Craig teaches methods of producing large numbers of compounds for testing against a number of biological targets.

In addition, Applicant would like to point out that the invention differs from the combination of cited references in that it teaches a cell-based screening assay wherein a ligand is contacted with the cells for binding ***intracellularly*** to biological components whose presence or amount is indicative of the intracellular biological or chemical process of interest. In addition, Applicant teaches an assay method whereby the biological component-associated ligand may be detected while still in the cell. In other words, the present invention has the advantage of not requiring cell lysis prior to detection. Furthermore, Applicant teaches a method of screening compounds that can affect post-translational events (newly added claims 68 and 69). It is generally known in the art that most cell-based assays have used reporter genes as an indicator of cellular activity (Silverman et al, "New assay technologies for high-throughput screening", *Curr.*

Opin. Chem. Biol., **2**, 397-403 (1998)), which reporter genes restrict the detection of cellular processes to transcriptional events. Also, although the changes in mRNA levels in a cell resulting from treatment with a small molecule can be used as a fingerprint as suggested by DeRisi *et al.* (DeRisi et al, "Exploring the metabolic and genetic control on gene expression on a genomic scale", Science, 278, 680-686 (1997)), many cellular events, including post-translational events, cannot be detected with this method. Applicant teaches an assay format capable of detecting post-translational events, which can therefore be used to provide a functional profile (newly added claim 81) for each test compound, and to classify compounds functionally (*e.g.*, according to the intracellular biological processes that they affect). Applicant submits that there is no suggestion or teaching in the cited references for a method of screening compounds that can affect intracellular post-translational events using a detecting ligand that binds to the target biological or chemical component intracellularly, nor is there any suggestion or teaching in the references to profile compounds according to the intracellular biological or chemical processes that they affect. In addition, the cited references do not teach or suggest the use of a ligand that binds to biological components intracellularly, or that ligands associated to the biological component might be detected inside the cells.

Accordingly, Applicant respectfully submits that a person of ordinary skill in the art would ***not*** have found "obvious to try" to combine the teachings of Gallop, Manns and Craig; Zambias, Manns and Craig; and/or Godowski, Manns and Craig, because there was no suggestion to combine the references, nor was there a reasonable expectation of success in the combinations in order to achieve the claimed invention. Specifically, there is no reasonable expectation of success that an intracellular product of a biological or chemical process can be detected by binding specifically to a ligand intracellularly. Applicant also submits that new claims 57-81 are not obvious over the combinations of cited references because the combinations of the cited references do not teach all of the claim limitations (*e.g.*, intracellular binding and detection of ligand, detection of post-translational events, functional fingerprinting). However, the present invention as described in the specification, particularly in the Examples, demonstrated experimentally that compounds ***can*** be screened for their ability to exert an effect

on intracellular biological or chemical processes in high-density cell-based assay format, whereby the effect is detected via intracellular binding of a ligand with biological components characteristic of the biological process of interest.

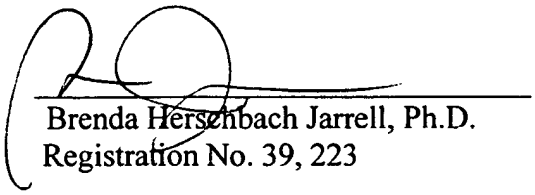
Since there is no reasonable expectation of success in combining the teachings of Gallop, Manns and Craig; Zambias, Manns and Craig; and Godowski, Manns and Craig to achieve the claimed invention, and since the references do not teach all of the claim limitations, claims 57-81 are not obvious over Gallop, Zambias and/or Godowski in view of Manns and in view of Craig.

Conclusion

In view of the foregoing arguments, Applicant respectfully submits that the claims are now in condition for allowance and thus respectfully requests withdrawal of the stated rejections. Applicant would like to thank the Examiner for his careful review and consideration of this case and if the Examiner believes that a telephone interview would be of assistance in advancing the prosecution of this application, the Examiner is invited to telephone the undersigned (617) 248-5175.

Please charge any fees that may be required for the processing of this Response, or credit any overpayments, to our Deposit Account No. 03-1721.

Respectfully submitted,



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Appendix
Version with Markings to Show Changes Made

Paragraph starting on page 4 line 5 and ending on page 4 line 13:

The present invention provides a system for the high throughput screening of chemical compounds. The system is particularly applicable to analysis of compounds that affect biological processes. In preferred embodiments, the invention detects events that occur inside cells. For example, the inventive system may be applied to the detection of compounds that alter the intracellular concentration of a target biological compound. Alternatively or additionally, the inventive system may be employed to identify compounds that suppress or enhance a specific biological phenotype. In preferred embodiments, the compounds analyzed comprise compounds synthesized by combinatorial chemistry.

Paragraph starting on page 5 line 17 and ending on page 5 line 23:

In one preferred embodiment, the present invention screens chemical compounds for their effects on chemical and/or biological systems by detecting the [present] presence or amount of a component present or produced by the system, in which the component acts as a marker for the chemical or biological process of interest. Preferably, the component is detected by means of its interaction with a binding partner ligand. Preferably, the binding is specific. In certain preferred embodiments, the binding partner ligand is an antibody.

Paragraph starting on page 6 line 17 and ending on page 7 line 6:

In another preferred embodiment, the present invention provides a system for identifying compounds capable of affecting a biological or chemical process comprising a high density array of reaction vessels containing at least 100 reaction vessels and an assay solution containing at least one reagent for detecting levels of component in a biological or a chemical process or resulting from a biological or a chemical process. Preferably, the array of reaction vessels contains at least 300 reaction vessels, and/or each vessel has a volume less than or equal to approximately 50 microliters, and/or the assay solution includes a component that is detected using [chemiluminesce] chemiluminescence. More preferably, the array of reaction vessels contains at least 1000 reaction vessels, each vessel has a volume less than or equal to

approximately 2 microliters, and/or the detected chemiluminescent compound is produced by a peroxidase. Most preferably, the array of reaction vessels contains at least 5000 reaction vessels, each vessel has a volume less than or equal to approximately 250 nanoliters, and/or the peroxidase is horseradish peroxidase.

Paragraph starting on page 7 line 15 and ending on page 7 line 17:

The present invention further provides compounds and compositions that are useful as microtubule stabilizers and/or as specific effectors of the cytoskeleton, [and] as well as methods for using such compounds and compositions.

Paragraph starting on page 28 line 17 and ending on page 28 line 23:

Preferably, the cells used in reaction vessels described in the preceding paragraph are mammalian cells. However, any biological or chemical system may be utilized in the reaction vessels in accordance with the present invention. For a non-limiting example of another biological system, other cells such as bacteria, yeast, plant and insect cells may be used. The number of cells for these [example] examples that are used in miniaturized reaction vessels will differ from mammalian cells depending on the size of the cells.

Paragraph starting on page 37 line 20 and ending on page 38 line 12 :

In one particular preferred embodiment of the present invention, the detectable entity comprises a peroxidase that catalyzes a chemiluminescent reaction. For example, a variety of chemiluminescent substrates are available for horse radish peroxidase (HRP). Preferred for use in the practice of the present invention are diacylhydrazides, such as luminol. Diacylhydrazides are oxidized in the presence of hydrogen peroxide, and luminesce to emit photons. The luminescence resulting from the oxidation of luminol can be enhanced using a phenol derivative, preferably 4-iodophenol (ECL™; Nycomed Amersham Corporation, Buckinghamshire, England). The luminescence can then be detected by film, detected using photomultiplier technology or detected by a charge-coupled device attached to a camera and/or a computer. The use of luminol as an HRP substrate greatly enhances the sensitivity of detecting HRP relative to other substrates such as color dyes (*e.g.* o-phenylenediamine; OPD). This increased sensitivity

of detection allows for small sample sizes. Figure 1 presents a schematic representation of but one particular preferred embodiment of the present invention, in which HRP is coupled to a secondary antibody, used to detect a primary antibody that interacts with a detection target.

Paragraph starting on page 69 line 16 and ending on page 69 line 22 :

We have used the inventive cytoblot system to identify compounds that alter the progression of mammalian cells through the cell division cycle. In particular, we have found one set of compounds that exibit the vinblastine-like property of destabilizing microtubules, one set of compounds that exhibit the taxol-like property of stabilizing microtubules, and one set of compounds that [alters] alter chromosome segregation in a novel fashion. Of particular interest are the microtubule destabilizing compounds.

Paragraph starting on page 69 line 23 and ending on page 70 line 9 :

In general, the present invention provides compounds and pharmaceutical compositions that alter the progression of cells through the cell cycle (see Figures 24-26). Compounds of particular interest are summarized in Figure 26. In certain preferred embodiments, the compounds and are capable of acting as inhibitors of the cell cycle. Specifically, these compounds are useful as microtubule stabilizers and as specific effectors of the cytoskeleton. In one aspect, the present invention provides novel compounds as shown by **(10)**, **(20)**, **(30)**, **(40)**, **(50)** and **(60)** below, and as described below. Furthermore, the present invention provides pharmaceutical compositions comprising a therapeutically effective amount of the compound having any one of the structures **(10)**, **(20)**, **(30)**, **(40)**, **(50)**, or **(60)**, associated with a pharmaceutically acceptable carrier.

Paragraph starting on page 75 line 4 and ending on page 75 line 16:

R_1-R_8 are each independently the same or different and are selected from the group consisting of H, Br, Cl, F, NH_2, CO_2H, OH, linear or branched alkyl, linear or branched acylamino, linear or branched acyloxy, linear or branched alkoxycarbonyl, linear or branched alkoxy, aryloxy, linear or branched alkylaryl, linear or branched hydroxyalkyl, and linear or branched aminoalkyl or aryl group. [Each of the] The abovedescribed compounds represent

novel compounds provided by the present invention, with the limitation that, in **(20)** above, R_1-R_8 cannot each simultaneously comprise hydrogen. Each of the abovedescribed compounds can be associated with a pharmaceutically acceptable carrier to provide novel pharmaceutical compositions, even when R_1-R_8 each simultaneously [comprise] comprises hydrogen. In a particularly preferred embodiment, compositions are provided where R_1-R_8 are each hydrogen. Each class of compounds, as depicted by **(10)** and **(20)** [above affect] above, affects the cell cycle by stabilizing microtubules.

Paragraph starting on page 81 line 9 and ending on page 81 line 10 :

Each of the compounds **(30)-(60)** shown above [are] is capable of interfering with the cytoskeletal structure of cells undergoing mitosis.

Paragraph starting on page 81 line 11 and ending on page 81 line 13 :

Furthermore, as will be appreciated by one of ordinary skill in the art, [the] the present invention is intended to include all enantiomers and diastereomers of the inventive compounds utilized in the compositions and methods.

Paragraph starting on page 81 line 16 and ending on page 82 line 2 :

The compounds disclosed herein inhibit cell cycle progression by either 1) acting on microtubules or 2) effecting the mitotic cytoskeleton, and thus may be used to treat a variety of human conditions including a broad range of cancers and pathogenic infections. As noted above microtubule stabilizing agents may be used to prevent or reduce atherosclerosis or restenosis. Furthermore, compounds of the present invention may be used as [immunosurpressants] immunosuppressants or as morning-after pills. Thus, the present invention provides pharmaceutical compositions comprising any one of the abovedescribed compounds **(10)**, **(20)**, **(30)**, **(40)**, **(50)**, or **(60)** and a pharmaceutically acceptable carrier. Methods for treating disorders are also provided comprising administering a therapeutically effective amount of an inventive composition to a patient in need.

Paragraph starting on page 84 line 5 and ending on page 84 line 18:

Clearly, compounds that act as cell cycle inhibitors are invaluable to the study of the cell cycle pathway. In general, inhibitors of cell cycle progression are essential as tools that can be used to achieve arrest at specific points in the cell cycle. This allows one to administer the reagent to a population of cells to achieve synchronization of the [mitutic] mitotic cell cycle. In addition, specific proteins or activities may be identified as being essential to cell-cycle-related functions by their interaction with small molecule inhibitors of the cell cycle. Proteins that play an important role downstream of the direct target may be confirmed by indirect inhibition by the same agent. In essence, exposure of cells to such reagents causes a conditional loss of function in the target protein in a similar manner to that achieved by the use of temperature-sensitive mutations in a gene. Similarly, such inhibitors of microtubule polymerization and depolymerization may be used to identify new cytoskeletal proteins and unravel the function and regulation of cytoskeletal proteins.

Paragraph starting on page 84 line 19 and ending on page 85 line 14:

Additionally, as will be appreciated by one of ordinary skill in the art, any in vitro assay may be used to monitor inhibition at different mitotic transition points, when the cell cycle progresses from one phase to the next. This may be accomplished by altering the timing of addition of the chemical compound in question to the mitotic extract. Alternatively it may be desirable to test whether certain compounds inhibit mitosis at early transition stages (*e.g.*, prophase or anaphase). According to one preferred embodiment of the present invention, the test compound is added to the interphase mitotic extract simultaneously with the Delta90 cyclin protein (and thus at the onset of mitosis) to test for successful of inhibition of early transition stages. Another aspect of the invention tests whether certain compounds inhibit mitosis at late transition stages (*e.g.*, microtubule assembly and disassembly and chromosome segregation). Thus according to other preferred embodiments of the present invention, the test compound is added after Delta90 cyclin so that mitosis has progressed past the early transition stages and inhibition of ubiquitin degradation can be assessed. Effectors of microtubule stability are particularly desirable compounds according to the present invention. Identification of such compounds [are] is likely to allow further dissection of key regulatory steps of the mitotic

pathway, cytoskeletal organization and serve as important tool in various other research and therapeutic purposes as mentioned above.

Paragraph starting on page 91 line 13 and ending on page 91 line 19 :

In agreement with the phenotypic effects of synstab A observed through [fluorscence] fluorescence microscopy, fluorescence-activated cell sorting confirmed that, similar to cells treated with [nicodazole] nocodazole or taxol, cells treated with synstab A had fully replicated chromosomes (4N DNA content) and increased TG-3 staining. In addition, immunoblotting of total cell extracts derived from cells treated with taxol or with [systab] synstab A at concentrations that do not affect viability show increased TG-3 reactivity.

Paragraph starting on page 92 line 7 and ending on page 92 line 24 :

We investigated the phenotype of [mamalian] mammalian cells (BS-C-I) treated with the compounds of group III . Live images were taken as described previously (Cramer *et al.*, *Curr. Op. Cell Biol.* 6:82, 1994); for [immunofluoresence] immunofluorescence, the cells were stained with a Golgi-specific antibody (anti-Golgi 58K protein antibodies [Sigma]) or with anti-α-tubulin antibodies (DM1 A [Sigma]); actin was visualized using [TRIC-conjugated] TRITC-conjugated phalliodin (Sigma); lysosomes were stained with LysoTracker (Molecular Probes; Palmiter *et al.*, *EMBO J.* 15:1784, 1996). Our examination of the distribution of microtubules, actin, and chromatin in fixed cells by fluorescence microscopy allowed us to divide the small molecules into three classes. Twenty-seven had no observable effect on the microtubule and actin cytoskeleton or on [cheomosome] chromosome distribution. Consistent with the data from the cytoblot assay we observed an increase in the number of normal appearing mitotic cells. These compounds may increase the mitotic index by perturbing the function of proteins that regulate progression through the cell cycle, *e.g.*, anaphase regulators, rather than structural or mechanochemical components of the mitotic spindle. It is also possible that these compounds have a subtle effect on cytoskeletal dynamics or chromosome organization that may not be observable in fixed cells.

Paragraph starting on page 96 line 9 and ending on page 97 line 6:

We refer to this approach of phenotype-based small molecule screening as chemical genetics, because of its conceptual similarity to classic forward genetic screens. The cytoblot assay will be a key tool for chemical genetics. Using appropriate antibodies, it can provide a quantitative readout of essentially any [post-transnational] post-translational modification of a specific protein in the cell. In this Example, a cytoblot assay for phosphorylation of nucleolin was used as a readout of mitosis, and our screen detected compounds that arrest cells in mitosis. After eliminating compounds that targeted pure tubulin, a sufficiently small number of the original 16,320 compounds remained for us to use a systematic visual analysis. For monastrol, the information from such analyses facilitated the identification of the kinesin Eg5 as a cellular target. Previously the only known small molecule kinesin inhibitors were 5'-adenylylimido-diphosphate (AMP-PNP) (Saxon, *Met. Cell Biol.* 44:279, 1994) and a marine natural product (Sakowicz *et al.*, *Science* 280:292, 1998), both of which are not cell-permeable and affect multiple kinesin family members. Monastrol, in contrast, is the first example of a cell-permeable compound that selectively perturbs the function of a motor protein essential for mitosis. Other motor proteins involved in lysosome and Golgi distribution seem not to be affected by other mechanisms have shown anti-tumor activity in humans (Jordan *et al.*, *Met. Enzymol.* 298:252, 1998), monastrol may serve as a lead for anti-cancer drugs. Monastrol will, however, be a valuable tool for dissecting the function of Eg5 in the establishment of spindle bipolarity and other cellular processes.

Paragraph starting on page 102 line 14 and ending on page 103 line 17:

LUCIFERASE ASSAYS: Our transient transfection luciferase assay was described previously by us (Stockwell *et al.*, Chem Biol 5:385, 1998). Briefly, 100,000 Mv1Lu mink lung epithelial cells were transiently transfected in 12-well dishes with 400 ng p3TPLux or pNFkB-Lux, with or without 50 ng pFC-MEKK, in 300 μL minimal essential medium with non-essential amino acids. The DEAE-dextran/chloroquine/DMSO [methods] method was used for transfection (Stockwell *et al.*, Chem Biol 6:71, 1999). After cell lysis in 120 μL lysis buffer, a Beckman LS 6500 liquid scintillation counter was used in single photon mode to quantitate luminescence. For detection of luciferase activity in 6F cells (including the primary screen), 20,000 6F cells were seeded in 50 μL of 10% mink medium in each well of a white 384-well plate (Nalge Nune International;

Naperville, IL; cat#164610) using a Multidrop 384 liquid dispenser (Lab Systems; Helsinki, Finland). After 16 hours, medium was removed using a 24 channel wand (V&P Scientific, Inc.; San Diego, CA; cat#VP186L), the cells were washed with 75 μL of 0.2% mink medium (containing 0.2% FBS), and reagents were added in 40 μL of 0.2% medium. For the primary screen, reagents were added by pin transfer using 384 polypropylene pin arrays (Matrix Technologies; Hudson, NH). After 24 hours, the cells were cooled on ice and washed twice with 75 μL Hanks Balanced Salt Solution (HBSS; GibcoBRL; cat#24020-117). Then 20 μL lysis buffer (25 mM glycylglycine (Sigma; cat#E-0396), 1% Triton X-100 (Sigma; cat#T-9284), 1mM dithiothreitol (DTT; Sigma; cat#D-5545), 1mM phenylmethylsulfonyl fluoride (PMSF;Sigma; cat#P-7626)) was added to each well with a Multidrop. After incubating the cells for five minutes on ice, 20 μL of ATP/luciferin solution was added (25 mM glycylglycine pH 7.8, 15mM $MgSO_4$, 4 mM EGTA, 6.25 mM K_2HPO_4 (Sigma; cat#P5504) pH 7.8, 5mM DTT, 75 μM D-luciferin (Sigma, cat# L-9504, 2mM ATP (Sigma; cat#A-7699)). Light output was immediately measured with an Analyst 384-well platereader (LJL), with 0.5 second counting time per well.

New assay technologies for high-throughput screening

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The use of high-throughput screening for early stage drug discovery imposes several constraints on the format of assays for therapeutic targets of interest. Homogeneous cell-free assays based on energy transfer, fluorescence polarization spectroscopy or fluorescence correlation spectroscopy provide the sensitivity, ease, speed and resistance to interference from test compounds needed to function in a high-throughput screening mode. Similarly, novel cell-based assays are now being adapted for high-throughput screening, providing for *in situ* analysis of a variety of biological targets. Finally, recent advances in assay miniaturization mark a transition to ultra high-throughput screening, ensuring that identification of lead compounds will not be the rate-limiting step in finding new drugs.

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Abbreviations

CRE	cAMP response element
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
HTS	high-throughput screening

Introduction

Continuing advances in molecular biology, human genetics and genomics have accelerated identification of the mechanisms underlying a growing number of human diseases. This progress has increased the number of novel protein targets available for potential therapeutic intervention by drug treatment. Concurrently, novel approaches in combinatorial chemistry and expanded collections of natural products have dramatically increased the number of compounds that can be tested for activity against these targets. The confluence of these two trends towards more potential targets and larger chemical libraries has greatly stimulated adoption of high-throughput screening (HTS) as the primary tool for early stage drug discovery.

HTS is the process by which large numbers of compounds are tested, in an automated fashion, for activity as inhibitors or activators of a particular biological target, such as a cell surface receptor or a metabolic enzyme. Although any assay performed on the bench top can, in theory, be applied in HTS, conversion to an automated format imposes certain constraints that affect the design of the assay in practice. Procedures that are routine at the bench are often extremely difficult to automate. Also, the more steps required for an assay, the more difficult to automate the HTS. The ideal assay is one that can be performed in a single well with no other manipulation other than addition of the sample to be tested.

A number of assay formats have been developed or modified over the past few years to conform to the constraints imposed by HTS. These assay protocols can be divided into two groups: cell-free assays that measure the biological activity of a relatively pure protein target and cell-based assays that assess the activity of a target protein by monitoring a biological response of a cell in which the target protein resides. In either case, the protocols require minimal manipulations, can be performed robotically in relatively small volumes, yield robust responses and are relatively impervious to perturbation by solvents and compounds used in drug screening. In this review we describe several of the more recently developed or exploited assay protocols for HTS.

Cell-free assays

The primary goal in adapting cell-free assays to HTS is to minimize the number of steps required in setting up the assay and in detecting the activity, be it an enzymatic reaction or the binding of two components. This goal has been met to a large extent by development of detection systems that do not require separation of the product of the reaction from substrate, or from other components of the assay mixture. Earlier approaches to such homogeneous assay formats relied on proximity-dependent energy transfer. The output of such assays derived from the signal enhancement generated by bringing a source and a distance-dependent amplifier close together. For example, the β-particles of a low-energy radionuclide attached to a ligand will stimulate the fluorescent emission of a scintillant in a bead to which the ligand's receptor is attached [1,2]. More recently, this detection method has been applied to enzymatic reactions, such as that catalyzed by topoisomerase I [3]. As another example of energy transfer assay formats, the rare earth metal lanthanide, Eu^{2+}, when irradiated by light, can transfer its excitation energy in a nonradiative process to the fluorescent protein, allophycocyanin, if the two are in close proximity. This can occur when a Eu^{2+}-derivitized ligand binds to an allophycocyanin-linked receptor [4,5] or a Eu^{2+}-derivitized anti-phosphotyrosine antibody binds to a detector-linked phosphorylated substrate of a tyrosine kinase such as src [6°]. Use of time resolved fluorescent procedures assessing emission at specific times following excitation enhances the sensitivity of this technique by reducing interference from background fluorescence, from test compounds or from assay components [6°,7°]. Finally, enzymatic assays suitable for HTS and based on fluorescent resonant energy

transfer between two different forms of green fluorescent protein (GFP) have recently been described [8•].

A number of investigators have exploited fluorescence polarization spectroscopy (FPS) as the basis for homogeneous HTS assays of both enzymatic and binding reactions. When fluorescent molecules in solution are excited with polarized light, the degree to which the emitted light retains polarization depends on the extent to which the fluorescent molecule rotates during the interval between excitation and emission. The rapid rotation of small fluorescent molecules in solution results in substantial loss of polarization. If such small molecules bind to larger molecules, their rotational diffusion is reduced and the retention of polarization is correspondingly increased. Thus, by measuring the relative intensity of emitted light in the planes normal and orthogonal to the plane of the incident polarized light, the extent of rotation of a target molecule, and inferentially, the extent of binding of the target molecule to a larger component, can be calculated. For instance, fluorescent polarization has been used to detect the presence of specific drugs or hormones [9,10], to assess antibody binding of fluorescein-conjugated peptides [11] or to monitor DNA : DNA hybrid formation [12]. The recent availability of a 96-well plate reader [13] with a high sensitivity to fluorescein and fluorescein conjugates has allowed development of 96-well based fluorescent polarization assays. Such high-throughput assays for src family tyrosine kinase activity [14•], for binding of phosphopeptides to Src SH2 domains [15•], for interaction between STAT1 and an γ-interferon receptor-derived phosphotyrosine-containing peptide [16•] and for specific protease activities [17,18•] have recently been described. The sensitivity of fluorescence polarization, the ease and speed with which such assays can be run and the resistance of such assays to interference from absorptive compounds commonly present in complex mixtures [18•] make this procedure highly amenable to HTS.

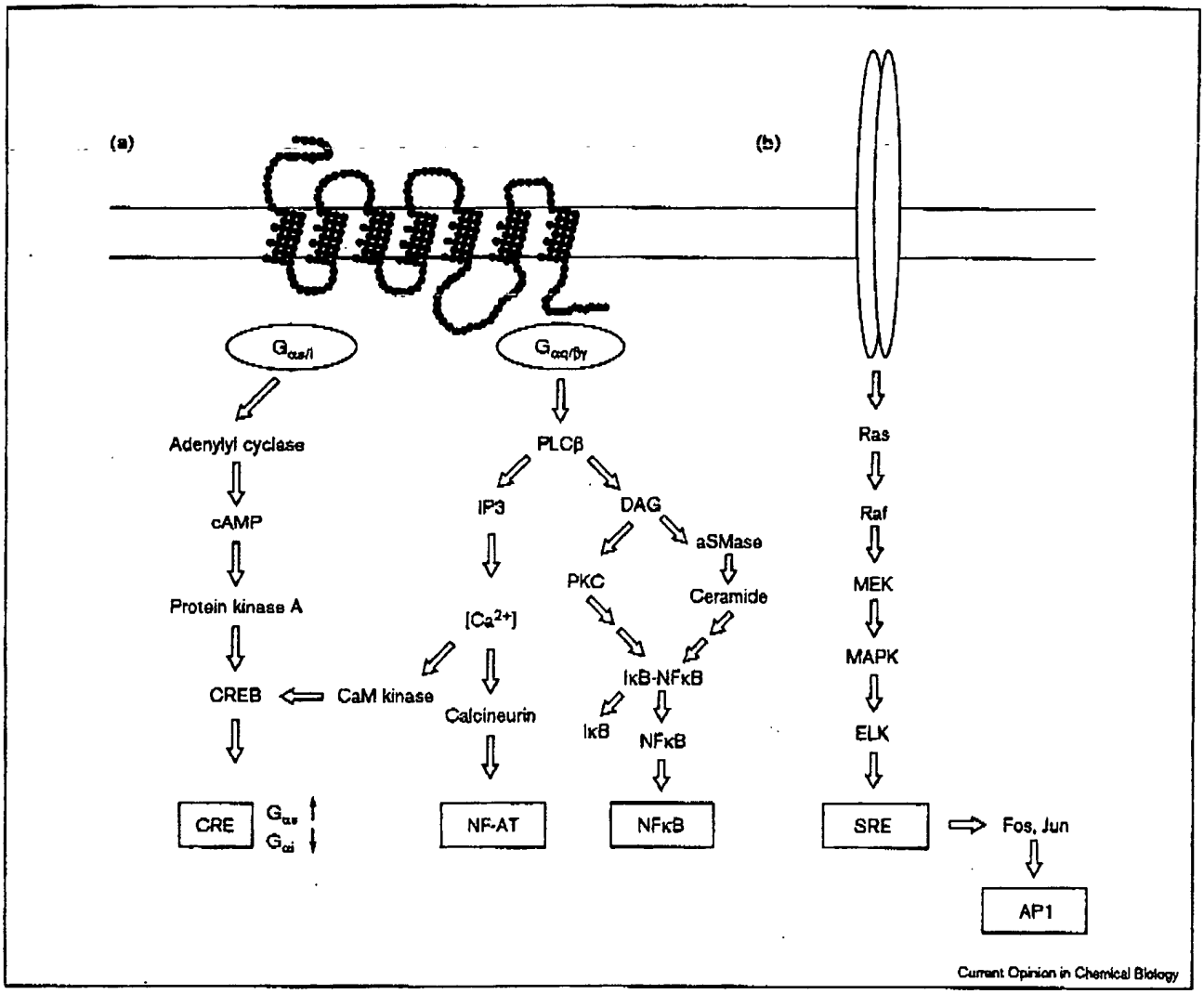
Fluorescence correlation spectroscopy (FCS) represents another recently developed detection format eminently suitable for HTS. FCS measures differences in physical states of a target molecule, such as bound versus free or cleaved versus intact, in a homogeneous mixture [19]. Specifically, FCS measures the burst of fluorescent emission of a molecule passing through a small volume of space, which is defined by a sharply focused laser beam. Small molecules diffuse through the volume rapidly and thus yield short bursts of light. Binding of these small molecules to larger molecules reduces their translational diffusion and correspondingly increases the duration of the bursts of light. Deconvolution of the emission patterns in a sample by appropriate software can yield the relative amount of the bound and unbound states of a fluorescently tagged ligand. This technology can therefore readily be applied to measure receptor–ligand interactions, DNA–protein interactions, nucleic acid hybrid formation and certain enzymatic reactions [20].

Cell-based assays

Cell-based assays are an increasingly attractive alternative to *in vitro* biochemical assays for HTS. Such *in vivo* assays require an ability to examine a specific cellular process and a means to measure its output. For instance, agonist activation of a cell surface receptor or a ligand-gated ion channel can elicit a change in the transcription pattern of a number of genes. This ligand-induced alteration in transcription can be readily captured by using gene fusions, in which a promoter element responsive to receptor activation is fused to the coding region for an enzyme or protein whose levels can be easily measured. Appreciation of the particular signaling pathway associated with a specific receptor allows identification of the appropriate transcriptional response element required to detect a response. Figure 1 depicts a number of signal transduction pathways, indicating the transcriptional response elements coupled to each pathway. Several reporter genes that generate products that can be adapted to HTS format are available [21,22]. These are listed in Table 1, with references to recent innovations in their use [23•,24,25,26•]. For instance, the recent report of novel fluorescent, cell-permeable substrates for β-lactamase documents the use of β-lactamase to detect receptor activation in single cells, making it an attractive assay system for high density HTS [27••].

While cell-based assays using reporter genes have proved effective as an HTS format, detecting more immediate responses to target protein activation provides several advantages, including shorter duration of the assay and fewer false positives from nonspecific interactions. As indicated in Figure 1, such cellular response dependent on activation of a receptor include elevation of a second messenger (for example, Ca^{2+}, cAMP, inositol triphosphate), phosphorylation of an intermediate signaling protein, or subcellular translocation of a signaling molecule. Recent advances in molecular biology and in instrumentation have made it possible to monitor these events in an automated format. For instance, the recent availability of a 96-well fluorescent imaging plate reader (Molecular Devices, Sunnyvale, California, USA) permits HTS of receptor activation by monitoring Ca^{2+} mobilization of cells preloaded with a fluorescent calcium indicator, such as FLUO-3 (Molecular Probes, Eugene, Oregon, USA). In addition, recombinant cells expressing a calcium-sensitive fluorescent protein, such as aequorin [28•] or a hybrid calmodulin–GFP protein [29••], obviate the need for preloading cells with dyes in order to detect calcium fluxes following stimulation. A separate approach to detecting early events following receptor stimulation involves examining relocalization of specific components of the signal transduction machinery. For instance, MAP kinase (Figure 1) relocalizes from the cytoplasm to the nucleus within minutes following stimulation of an upstream G-protein-coupled receptor [30,31]. Similarly, Barak *et al.* [32•] have shown that recruitment of a β-arrestin–GFP fusion protein to the plasma membrane can be used to monitor activation

Figure 1



Signal transduction pathways commonly used in mammalian cell-based high-throughput assays. **(a)** Agonist-engaged seven transmembrane receptors are functionally linked to the modulation of several well characterized enhancer/promoter elements, the cAMP response element (CRE), nuclear factor of activated T cells (NF-AT), NFκB, serum response element (SRE) and AP1 [46–49]. Upon activation of a $G_{\alpha s}$ coupling receptor, adenylyl cyclase is stimulated, producing increased concentrations of intracellular cAMP, stimulation of protein kinase A, phosphorylation of the CRE binding protein (CREB) and induction of promoters with CRE elements. $G_{\alpha i}$ coupling receptors dampen CRE activity by inhibition of the same signal transduction components. $G_{\alpha q}$ coupling receptors and some βγ pairs stimulate phospholipase C (PLC), and the generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). A transient flux in intracellular calcium promotes induction of calcineurin and NF-AT, as well as calmodulin (CaM)-dependent kinase and CREB. Increased DAG concentrations stimulate protein kinase C (PKC) and endosymal/lysosomal acidic sphingomyelinase (aSMase); while the aSMase pathway is dominant, both induce degradation of the NFκB inhibitor IκB as well as NFκB activation. By a poorly understood mechanism, IκB degradation may also be initiated through the MAPK (mitogen-activated protein kinase) cascade (not shown). **(b)** Growth factor receptor (depicted by ellipses) activation results in recruitment of Sos (not shown) to the plasma membrane, where it stimulates Ras, which recruits the serine/threonine kinase Raf to the plasma membrane. Once activated, Raf phosphorylates MEK kinase, which phosphorylates and activates MAPK and the transcription factor ELK (Ets-like protein, also known as p62 TCF1 [ternary complex factor 1]). ELK drives transcription from promoters with SRE elements, leading to synthesis of the transcription factors Fos and Jun, that form a transcription complex capable of activating AP1 sites. Seven transmembrane receptors also stimulate the MAPK pathway through βγ subunits, most probably through phosphoinositide 3-kinase γ (PI3Kγ; not shown).

of a number of different G-protein-coupled receptors. Recent advances in microscopic imaging technology, in conjunction with software permitting automated image recognition, provide a means to capture these events in a high-throughput mode.

Cell-based assays have significant advantages over *in vitro* assays. First, the starting material (the cell) self-replicates, avoiding the investment involved in preparing a purified target, in chemically modifying the target to suit the screen, and so on. Second, the targets and readouts are ex-

Table 1

Reporter genes useful for cell-based high-throughput screening.

Reporter genes (source)	Advantages	Disadvantages	References
β-galactosidase (bacterial)	Well characterized; stable, inexpensive substrates; highly sensitive fluorescent or chemiluminescent substrates available; little interference from test compounds; simple readouts (readily automated)	Endogenous activity (mammalian cells); tetrameric (non-linear response at low concentration)	[23•,50]
Luciferase (firefly)	Dimeric; high specific activity; no endogenous activity (low background)	Requires addition of cofactor (luciferin) and presence of O_2 and ATP	[23•]
Alkaline phosphatase (human placental)	Secreted protein (avoids the need for membrane-permeable substrates); inexpensive colorimetric and highly sensitive luminescent assays available	Endogenous activity in some cell types; optimal at pH 9.8	[24,25]
β-lactamase (bacterial)	Monomeric; highly sensitive fluorogenic substrates described; no endogenous activity	Membrane-permeable fluorescent substrates not readily available	[27••]
GFP (jellyfish)	Monomeric; no substrate needed (no manipulations required for assay); no endogenous activity; multiple forms available	Relatively low specific activity	[26•,51,52]

amined in a biological context that more faithfully mimics the normal physiological situation. Third, cell-based assays can provide insights into bioavailability and cytotoxicity. Mammalian cells are expensive to culture and difficult to propagate in the automated systems used for HTS, however.

An alternative to mammalian cell based assays is to recapitulate the desired human physiological process in a micro-organism such as yeast [33]. For instance, signaling via human G-protein-coupled receptors has been reconstituted in yeast to yield a facile growth response or a reporter gene readout ([34,35]; Klein *et al.*, unpublished data). Similarly, mammalian ion channels have been coupled to growth response in yeast [36]. Also, protein–protein interactions, including RAS–RAF association [37] and tyrosine kinase receptor–ligand binding [38], have been faithfully reproduced using the yeast two-hybrid system. Finally, many mammalian transcription factors operate in yeast, including glucocorticoid receptor [39,40] and the retinoic acid receptor and retinoid X receptor families of receptors [41]. The ease and low cost of growing yeast, their ready genetic manipulation, and their resistance to solvents make yeast an attractive option for cell-based HTS.

Miniaturization

Several factors are fueling efforts to increase the speed of HTS and decrease the volume of individual reactions within an HTS format. Split-bead synthesis (see Note added in proof), or other similar approaches to combinatorial chemistry, dramatically increases the number of compounds that can be produced in a library but do so at the cost of quantity of material. In addition, the limited supply of existing compounds within chemical libraries of pharmaceutical companies, and the growing number of targets against which such compounds can be tested, motivate a frugal approach to use of those compounds. Finally, the reagent costs associated with HTS, when multiplied by the increasing number of assays per run, are becoming a significant cost of early stage drug discovery.

In response to these exigencies, a number of groups have begun to develop formats for very high density screening using very small assay volumes. One approach involves reducing the well size and increasing the density of the assay plate but retaining the overall assay format used in current 96-well based HTS. Densities of 6500 assays in a 10 cm array have been reported for cell-free enzyme based assays [42•] and for ligand binding in cell based assays [43••]. This approach of miniaturizing existing formats significantly increases the number of assays per plate and the overall throughput of the screen but is intrinsically limited by the physical constraints of delivering small volumes to wells, and of detecting responses in a sensitive and timely manner. Accordingly, novel formats have been developed that eschew the assay format based on wells. One approach uses glass chips containing microchannels in which reagents, target proteins and compounds are herded by electrokinetic flow controlled by electric potentials applied at the ends of the channels [44•]. A related approach attains high-throughput both of chemical synthesis and activity assessment by parallel arrays of three-dimensional channels in which flow is controlled by miniature hydrostatic actuators [45]. These approaches provide significant reduction in the volume of assays and a corresponding savings in reagent costs over conventional HTS [45]. In addition, with further development in parallel processing in multiple chips, the number of assays performed in a given period

of time can increase dramatically. This movement to miniaturization is likely to ensure that the initial stage of drug discovery identification of lead compounds will not be the rate-limiting step in finding new drugs.

Conclusions

The last decade has witnessed the emergence across the pharmaceutical industry of the 96-well-based, robotics-driven, high-throughput screening process as the primary tool for identifying active compounds in the first stage of drug discovery. This program has dictated the format of the assays that are used to assess the activities of targets — enzymes, receptors, transporters and so on — that underlie drug discovery in various therapeutic areas. A number of such formats — resonant energy transfer and fluorescent polarization spectroscopy in cell-based assays — have gained widespread acceptance and growing incorporation into high-throughput screening programs. The growing number of potential therapeutic targets, the increasing number of screenable compounds, the accelerating costs of screening and the increasing pressure to generate more lead compounds in a shorter time all conspire to render even the new approaches inadequate for meeting the anticipated throughput requirements, however. Thus, we are likely to witness a movement towards even greater screening throughput by miniaturization and increased reliance on robotics. Whether a new standard format for screening emerges in the near future, or whether a variety of formats are pursued concurrently remains to be seen. Nonetheless, we can anticipate that the exigencies of drug screening will motivate a continued application of state-of-the-art technologies to the process of high-throughput screening.

Note added in proof

For a reference describing split-bead synthesis, see [53].

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- •• of outstanding interest

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Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale

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DNA microarrays containing virtually every gene of *Saccharomyces cerevisiae* were used to carry out a comprehensive investigation of the temporal program of gene expression accompanying the metabolic shift from fermentation to respiration. The expression profiles observed for genes with known metabolic functions pointed to features of the metabolic reprogramming that occur during the diauxic shift, and the expression patterns of many previously uncharacterized genes provided clues to their possible functions. The same DNA microarrays were also used to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*. These results demonstrate the feasibility and utility of this approach to genomewide exploration of gene expression patterns.

The complete sequences of nearly a dozen microbial genomes are known, and in the next several years we expect to know the complete genome sequences of several metazoans, including the human genome. Defining the role of each gene in these genomes will be a formidable task, and understanding how the genome functions as a whole in the complex natural history of a living organism presents an even greater challenge.

Knowing when and where a gene is expressed often provides a strong clue as to its biological role. Conversely, the pattern of genes expressed in a cell can provide detailed information about its state. Although regulation of protein abundance in a cell is by no means accomplished solely by regulation of mRNA, virtually all differences in cell type or state are correlated with changes in the mRNA levels of many genes. This is fortuitous because the only specific reagent required to measure the abundance of the mRNA for a specific gene is a cDNA sequence. DNA microarrays, consisting of thousands of individual gene sequences printed in a high-density array on a glass microscope slide (*1*, *2*), provide a practical and economical tool for studying gene expression on a very large scale (*3*–*6*).

Saccharomyces cerevisiae is an especially favorable organism in which to conduct a systematic investigation of gene expression. The genes are easy to recognize in the genome sequence, *cis* regulatory elements are generally compact and close to the transcription units, much is already known about its genetic regulatory mechanisms, and a powerful set of tools is available for its analysis.

A recurring cycle in the natural history of yeast involves a shift from anaerobic (fermentation) to aerobic (respiration) metabolism. Inoculation of yeast into a medium rich in sugar is followed by rapid growth fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (*7*). We used DNA microarrays to characterize the changes in gene expression that take place during this process for nearly the entire genome, and to investigate the genetic circuitry that regulates and executes this program.

Yeast open reading frames (ORFs) were amplified by the polymerase chain reaction (PCR), with a commercially available set of primer pairs (*8*). DNA microarrays, containing approximately 6400 distinct DNA sequences, were printed onto glass slides by using a simple robotic printing device (*9*). Cells from an exponentially growing culture of yeast were inoculated into fresh medium and grown at 30°C for 21 hours. After an initial 9 hours of growth, samples were harvested at seven successive 2-hour intervals, and mRNA was isolated (*10*). Fluorescently labeled cDNA was prepared by reverse transcription in the presence of Cy3(green)- or Cy5(red)-labeled deoxyuridine triphosphate (dUTP) (*11*) and then hybridized to the microarrays (*12*). To maximize the reliability with which changes in expression levels could be discerned, we labeled cDNA prepared from cells at each successive time point with Cy5, then mixed it with a Cy3-labeled "reference" cDNA sample prepared from cells harvested at the first interval after inoculation. In this experimental design, the relative fluorescence intensity measured for the Cy3 and Cy5 fluors at each array element provides a reliable measure of the relative abundance of the corresponding mRNA in the two cell populations (Fig. 1). Data from the series of seven samples (Fig. 2), consisting of more than 43,000 expression-ratio measurements, were organized into a database to facilitate efficient exploration and analysis of the results. This database is publicly available on the Internet (*13*).

During exponential growth in glucose-rich medium, the global pattern of gene expression was remarkably stable. Indeed, when gene expression patterns between the first two cell samples (harvested at a 2-hour interval) were compared, mRNA levels differed by a factor of 2 or more for only 19 genes (0.3%), and the largest of these differences was only 2.7-fold (*14*). However, as glucose was progressively depleted from the growth media during the course of the experiment, a marked change was seen in the global pattern of gene expression. mRNA levels for approximately 710 genes were induced by a factor of at least 2, and the mRNA levels for approximately 1030 genes declined by a factor of at least 2. Messenger RNA levels for 183 genes increased by a factor of at least 4, and mRNA levels for 203 genes diminished by a factor of at least 4. About half of these differentially expressed genes have no currently recognized function and are not yet named. Indeed, more than 400 of the differentially expressed genes have no apparent homology

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to any gene whose function is known (*15*). The responses of these previously uncharacterized genes to the diauxic shift therefore provides the first small clue to their possible roles.

The global view of changes in expression of genes with known functions provides a vivid picture of the way in which the cell adapts to a changing environment. Figure 3 shows a portion of the yeast metabolic pathways involved in carbon and energy metabolism. Mapping the changes we observed in the mRNAs encoding each enzyme onto this framework allowed us to infer the redirection in the flow of metabolites through this system. We observed large inductions of the genes coding for the enzymes aldehyde dehydrogenase (*ALD2*) and acetyl–coenzyme A(CoA) synthase (*ACS1*), which function together to convert the products of alcohol dehydrogenase into acetyl-CoA, which in turn is used to fuel the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. The concomitant shutdown of transcription of the genes encoding pyruvate decarboxylase and induction of pyruvate carboxylase rechannels pyruvate away from acetaldehyde, and instead to oxalacetate, where it can serve to supply the TCA cycle and gluconeogenesis. Induction of the pivotal genes *PCK1*, encoding phosphoenolpyruvate carboxykinase, and *FBP1*, encoding fructose 1,6-biphosphatase, switches the directions of two key irreversible steps in glycolysis, reversing the flow of metabolites along the reversible steps of the glycolytic pathway toward the essential biosynthetic precursor, glucose-6-phosphate. Induction of the genes coding for the trehalose synthase and glycogen synthase complexes promotes channeling of glucose-6-phosphate into these carbohydrate storage pathways.

Just as the changes in expression of genes encoding pivotal enzymes can provide insight into metabolic reprogramming, the behavior of large groups of functionally related genes can provide a broad view of the systematic way in which the yeast cell adapts to a changing environment (Fig. 4). Several classes of genes, such as cytochrome c–related genes and those involved in the TCA/glyoxylate cycle and carbohydrate storage, were coordinately induced by glucose exhaustion. In contrast, genes devoted to protein synthesis, including ribosomal proteins, tRNA synthetases, and translation, elongation, and initiation factors, exhibited a coordinated decrease in expression. More than 95% of ribosomal genes showed at least twofold decreases in expression during the diauxic shift (Fig. 4) (*13*). A noteworthy and illuminating exception was that the genes encoding mitochondrial ribosomal genes were generally induced rather than repressed after glucose limitation, highlighting the requirement for mitchondrial biogenesis (*13*). As more is learned about the functions of every gene in the yeast genome, the ability to gain insight into a cell's response to a changing environment through its global gene expression patterns will become increasingly powerful.

Several distinct temporal patterns of expression could be recognized, and sets of genes could be grouped on the basis of the similarities in their expression patterns. The characterized members of each of these groups also shared important similarities in their functions. Moreover, in most cases, common regulatory mechanisms could be inferred for sets of genes with similar expression profiles. For example, seven genes showed a late induction profile, with mRNA levels increasing by more than ninefold at the last timepoint but less than threefold at the preceding timepoint (Fig. 5B). All of these genes were known to be glucose-repressed, and five of the seven were previously noted to share a common upstream activating sequence (UAS), the carbon source response element (CSRE) (*16–20*). A search in the promoter regions of the remaining two genes, *ACR1* and *IDP2*, revealed that *ACR1*, a gene essential for *ACS1* activity, also possessed a consensus CSRE motif, but interestingly, *IDP2* did not. A search of the entire yeast genome sequence for the consensus CSRE motif revealed only four additional candidate genes, none of which showed a similar induction.

Examples from additional groups of genes that shared expression profiles are illustrated in Fig. 5, C through F. The sequences upstream of the named genes in Fig. 5C all contain stress response elements (STRE), and with the exception

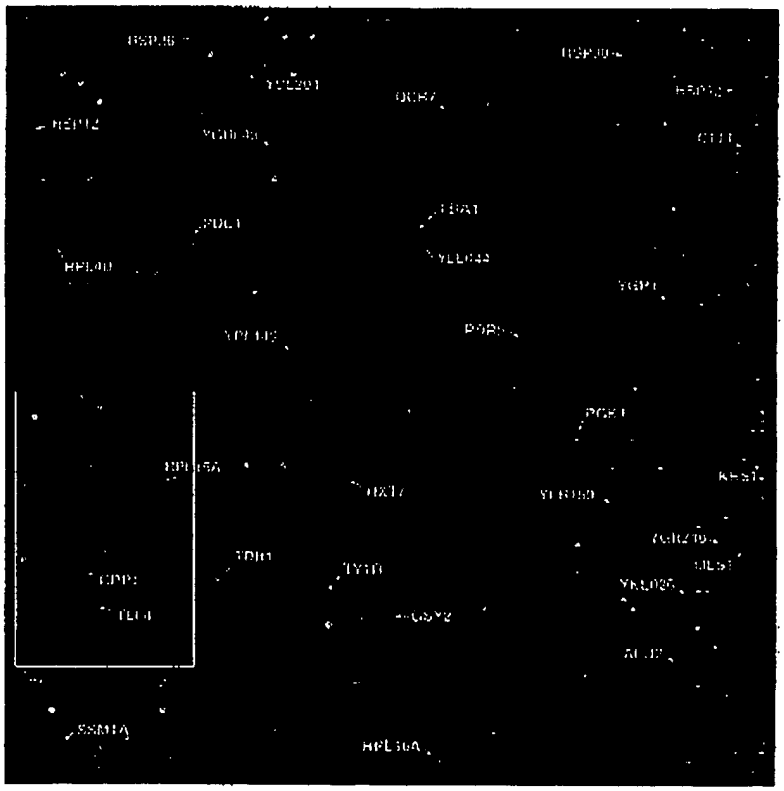

Fig. 1. Yeast genome microarray. The actual size of the microarray is 18 mm by 18 mm. The microarray was printed as described (*9*). This image was obtained with the same fluorescent scanning confocal microscope used to collect all the data we report (*49*). A fluorescently labeled cDNA probe was prepared from mRNA isolated from cells harvested shortly after inoculation (culture density of $<5 \times 10^6$ cells/ml and media glucose level of 19 g/liter) by reverse transcription in the presence of Cy3-dUTP. Similarly, a second probe was prepared from mRNA isolated from cells taken from the same culture 9.5 hours later (culture density of $\sim 2 \times 10^8$ cells/ml, with a glucose level of <0.2 g/liter) by reverse transcription in the presence of Cy5-dUTP. In this image, hybridization of the Cy3-dUTP–labeled cDNA (that is, mRNA expression at the initial timepoint) is represented as a green signal, and hybridization of Cy5-dUTP–labeled cDNA (that is, mRNA expression at 9.5 hours) is represented as a red signal. Thus, genes induced or repressed after the diauxic shift appear in this image as red and green spots, respectively. Genes expressed at roughly equal levels before and after the diauxic shift appear in this image as yellow spots.

of *HSP42*, have previously been shown to be controlled at least in part by these elements (*21–24*). Inspection of the sequences upstream of *HSP42* and the two uncharacterized genes shown in Fig. 5C, YKL026c, a hypothetical protein with similarity to glutathione peroxidase, and YGR043c, a putative transaldolase, revealed that each of these genes also possess repeated upstream copies of the stress-responsive CCCCT motif. Of the 13 additional genes in the yeast genome that shared this expression profile [including *HSP30*, *ALD2*, *OM45*, and 10 uncharacterized ORFs (*25*)], nine contained one or more recognizable STRE sites in their upstream regions.

The heterotrimeric transcriptional activator complex *HAP2,3,4* has been shown to be responsible for induction of several genes important for respiration (*26–28*). This complex binds a degenerate consensus sequence known as the CCAAT box (*26*). Computer analysis, using the consensus sequence TNRYTGGB (*29*), has suggested that a large number of genes involved in respiration may be specific targets of *HAP2,3,4* (*30*). Indeed, a putative *HAP2,3,4* binding site could be found in the sequences upstream of each of the seven cytochrome c–related genes that showed the greatest magnitude of induction (Fig. 5D). Of 12 additional cytochrome c–related genes that were induced, *HAP2,3,4* binding sites were present in all but one. Significantly, we found that transcription of *HAP4* itself was induced nearly ninefold concomitant with the diauxic shift.

Control of ribosomal protein biogenesis is mainly exerted at the transcriptional level, through the presence of a common upstream-activating element (UAS_{rpg}) that is recognized by the Rap1 DNA-binding protein (*31*, *32*). The expression profiles of seven ribosomal proteins are shown in Fig. 5F. A search of the sequences upstream of all seven genes revealed consensus Rap1-binding motifs (*33*). It has been suggested that declining Rap1 levels in the cell during starvation may be responsible for the decline in ribosomal protein gene expression (*34*). Indeed, we observed that the abundance of *RAP1* mRNA diminished by 4.4-fold, at about the time of glucose exhaustion.

Of the 149 genes that encode known or putative transcription factors, only two, *HAP4* and *SIP4*, were induced by a factor of more than threefold at the diauxic shift. *SIP4* encodes a DNA-binding transcriptional activator that has been shown to interact with Snf1, the "master regulator" of glucose repression (*35*). The eightfold induction of *SIP4* upon depletion of glucose strongly suggests a role in the induction of downstream genes at the diauxic shift.

Although most of the transcriptional responses that we observed were not previously known, the responses of many genes during the diauxic shift have been described. Comparison of the results we obtained by DNA microarray hybridization with previously reported results therefore provided a strong test of the sensitivity and accuracy of this approach. The expression patterns we observed for previously characterized genes showed almost perfect concordance with previously published results (*36*). Moreover, the differential expression measurements obtained by DNA microarray hybridization were reproducible in duplicate experiments. For example, the remarkable changes in gene expression between cells harvested immediately after inoculation and immediately after the diauxic shift (the first and sixth intervals in this time series) were measured in duplicate, independent DNA microarray hybridizations. The correlation coefficient for two complete sets of expression ratio measurements was 0.87, and for more than 95% of the genes, the expression ratios measured in these duplicate experiments differed by less than a factor of 2. However, in a few cases, there were discrepancies between our results and previous results, pointing to technical limitations that will need to be addressed as DNA microarray technology advances (*37*, *38*). Despite the noted exceptions, the high concordance between the results we obtained in these experiments and those of previous studies provides confidence in the reliability and thoroughness of the survey.

The changes in gene expression during this diauxic shift are complex and involve integration of many kinds of information about the nutritional and metabolic state of the cell. The large number of genes whose expression is altered and the diversity of temporal expression profiles observed in this experiment highlight the challenge of understanding the underlying regulatory mechanisms. One approach to defining the contributions of individual regulatory genes to a complex program of this kind is to use DNA microarrays to identify genes whose expression is affected

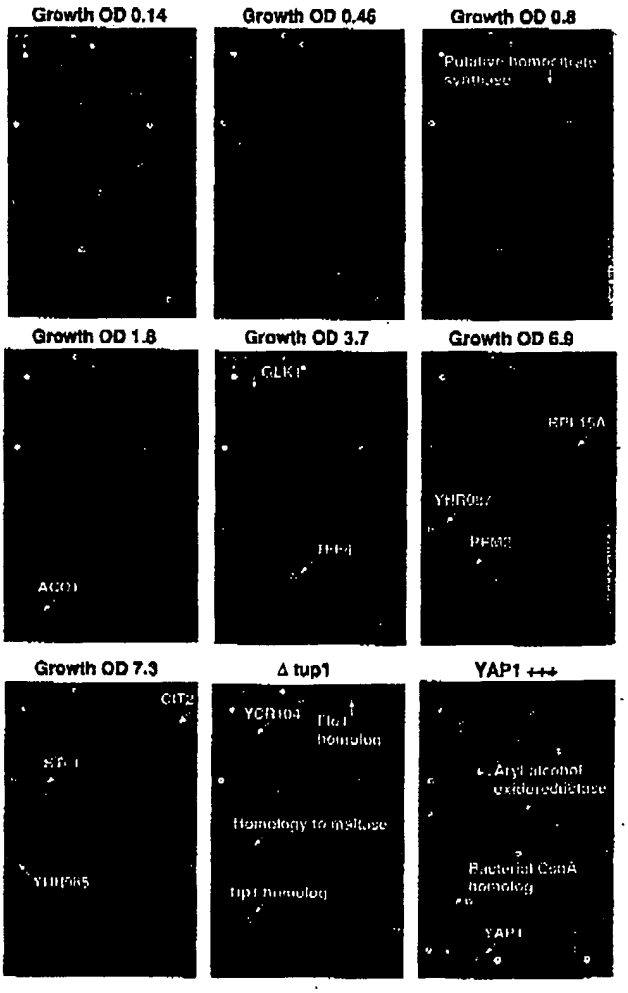

Fig. 2. The section of the array indicated by the gray box in Fig. 1 is shown for each of the experiments described here. Representative genes are labeled. In each of the arrays used to analyze gene expression during the diauxic shift, red spots represent genes that were induced relative to the initial timepoint, and green spots represent genes that were repressed relative to the initial timepoint. In the arrays used to analyze the effects of the *tup1*Δ mutation and *YAP1* overexpression, red spots represent genes whose expression was increased, and green spots represent genes whose expression was decreased by the genetic modification. Note that distinct sets of genes are induced and repressed in the different experiments. The complete images of each of these arrays can be viewed on the Internet (*13*). Cell density as measured by optical density (OD) at 600 nm was used to measure the growth of the culture.

by mutations in each putative regulatory gene. As a test of this strategy, we analyzed the genomewide changes in gene expression that result from deletion of the *TUP1* gene. Transcriptional repression of many genes by glucose requires the DNA-binding repressor Mig1 and is mediated by recruiting the transcriptional co-repressors Tup1 and Cyc8/Ssn6 (39). Tup1 has also been implicated in repression of oxygen-regulated, mating-type–specific, and DNA-damage–inducible genes (40).

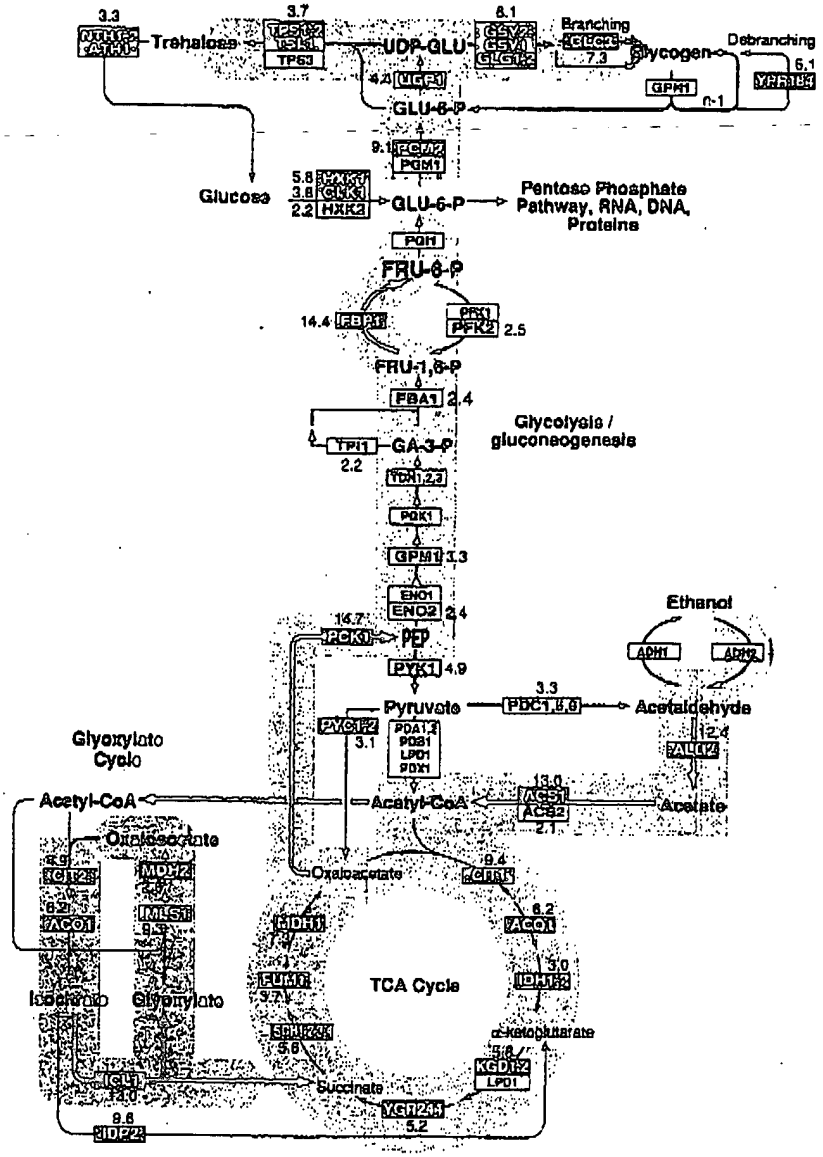

Fig. 3. Metabolic reprogramming inferred from global analysis of changes in gene expression. Only key metabolic intermediates are identified. The yeast genes encoding the enzymes that catalyze each step in this metabolic circuit are identified by name in the boxes. The genes encoding succinyl-CoA synthase and glycogen-debranching enzyme have not been explicitly identified, but the ORFs YGR244 and YPR184 show significant homology to known succinyl-CoA synthase and glycogen-debranching enzymes, respectively, and are therefore included in the corresponding steps in this figure. Red boxes with white lettering identify genes whose expression increases in the diauxic shift. Green boxes with dark green lettering identify genes whose expression diminishes in the diauxic shift. The magnitude of induction or repression is indicated for these genes. For multimeric enzyme complexes, such as succinate dehydrogenase, the indicated fold-induction represents an unweighted average of all the genes listed in the box. Black and white boxes indicate no significant differential expression (less than twofold). The direction of the arrows connecting reversible enzymatic steps indicate the direction of the flow of metabolic intermediates, inferred from the gene expression pattern, after the diauxic shift. Arrows representing steps catalyzed by genes whose expression was strongly induced are highlighted in red. The broad gray arrows represent major increases in the flow of metabolites after the diauxic shift, inferred from the indicated changes in gene expression.

Wild-type yeast cells and cells bearing a deletion of the *TUP1* gene (*tup1*Δ) were grown in parallel cultures in rich medium containing glucose as the carbon source. Messenger RNA was isolated from exponentially growing cells from the two populations and used to prepare cDNA labeled with Cy3 (green) and Cy5 (red), respectively (*11*). The labeled probes were mixed and simultaneously hybridized to the microarray. Red spots on the microarray therefore represented genes whose transcription was induced in the *tup1*Δ strain, and thus presumably repressed by Tup1 (*41*). A representative section of the microarray (Fig. 2, bottom middle panel) illustrates that the genes whose expression was affected by the *tup1*Δ mutation, were, in general, distinct from those induced upon glucose exhaustion [complete images of all the arrays shown in Fig. 2 are available on the Internet (*13*)]. Nevertheless, 34 (10%) of the genes that were induced by a factor of at least 2 after the diauxic shift were similarly induced by deletion of *TUP1*, suggesting that these genes may be subject to *TUP1*-mediated repression by glucose. For example, *SUC2*, the gene encoding invertase, and all five hexose transporter genes that were induced during the course of the diauxic shift were similarly induced, in duplicate experiments, by the deletion of *TUP1*.

The set of genes affected by Tup1 in this experiment also included α-glucosidases, the mating-type–specific genes *MFA1* and *MFA2*, and the DNA damage–inducible *RNR2* and *RNR4*, as well as genes involved in flocculation and many genes of unknown function. The hybridization signal corresponding to expression of *TUP1* itself was also severely reduced because of the (incomplete) deletion of the transcription unit in the *tup1*Δ strain, providing a positive control in the experiment (*42*).

Many of the transcriptional targets of Tup1 fell into sets of genes with related biochemical functions. For instance, although only about 3% of all yeast genes appeared to be *TUP1*-repressed by a factor of more than 2 in duplicate experiments under these conditions, 6 of the 13 genes that have been implicated in flocculation (*15*) showed a reproducible increase in expression of at least twofold when *TUP1* was deleted. Another group of related genes that appeared to be subject to *TUP1* repression encodes the serine-rich cell wall mannoproteins, such as Tip1 and Tir1/Srp1 which are induced by cold shock and other stresses (*43*), and similar, serine-poor proteins, the seripauperins (*44*). Messenger RNA levels for 23 of the 26 genes in this group were reproducibly elevated by at least 2.5-fold in the *tup1*Δ

strain, and 18 of these genes were induced by more than sevenfold when *TUP1* was deleted. In contrast, none of 83 genes that could be classified as putative regulators of the cell division cycle were induced more than twofold by deletion of *TUP1*. Thus, despite the diversity of the regulatory systems that employ Tup1, most of the genes that it regulates under these conditions fall into a limited number of distinct functional classes.

Because the microarray allows us to monitor expression of nearly every gene in yeast, we can, in principle, use this approach to identify all the transcriptional targets of a regulatory protein like Tup1. It is important to note, however, that in any single experiment of this kind we can only recognize those target genes that are normally repressed (or induced) under the conditions of the experiment. For instance, the experiment described here analyzed a *MAT* α strain in which *MFA1* and *MFA2*, the genes encoding the a-factor mating pheromone precursor, are normally repressed. In the isogenic *tup1Δ* strain, these genes were inappropriately expressed, reflecting the role that Tup1 plays in their repression. Had we instead carried out this experiment with a *MATA* strain (in which expression of *MFA1* and *MFA2* is not repressed), it would not have been possible to conclude anything regarding the role of Tup1 in the repression of these genes. Conversely, we cannot distinguish indirect effects of the chronic absence of Tup1 in the mutant strain from effects directly attributable to its participation in repressing the transcription of a gene.

Another simple route to modulating the activity of a regulatory factor is to overexpress the gene that encodes it. *YAP1* encodes a DNA-binding transcription factor belonging to the b-zip class of DNA-binding proteins. Overexpression of *YAP1* in yeast confers increased resistance to hydrogen peroxide, o-phenanthroline, heavy metals, and osmotic stress (*45*). We analyzed differential gene expression between a wild-type strain bearing a control plasmid and a strain with a plasmid expressing *YAP1* under the control of the strong *GAL1-10* promoter, both grown in galactose (that is, a condition that induces *YAP1* overexpression). Complementary DNA from the control and *YAP1* overexpressing strains, labeled with Cy3 and Cy5, respectively, was prepared from mRNA isolated from the two strains and hybridized to the microarray. Thus, red spots on the array represent genes that were induced in the strain overexpressing *YAP1*.

Of the 17 genes whose mRNA levels increased by more than threefold when *YAP1* was overexpressed in this way, five bear homology to aryl-alcohol oxidoreductases (Fig. 2 and Table 1). An additional four of the genes in this set also belong to the general class of dehydrogenases/oxidoreductases. Very little is known about the role of aryl-alcohol oxidoreductases in *S. cerevisiae*, but these enzymes have been isolated from ligninolytic fungi, in which they participate in coupled redox reactions, oxidizing aromatic, and aliphatic unsaturated alcohols to aldehydes with the production of hydrogen peroxide (*46*, *47*). The fact that a remarkable fraction of the targets identified in this experiment belong to the same small, functional group of oxidoreductases suggests that these genes might play an important protective role during oxidative stress. Transcription of a small number of genes was reduced in the strain overexpressing Yap1. Interestingly, many of these genes encode sugar permeases or enzymes involved in inositol metabolism.

We searched for Yap1-binding sites (TTACTAA or TGACTAA) in the sequences upstream of the target genes we identified (*48*). About two-thirds of the genes that were induced by more than threefold upon Yap1 overexpression had one or more binding sites within 600 bases upstream of the start codon (Table 1), suggesting that they are directly regulated by Yap1. The absence of canonical Yap1-bind-

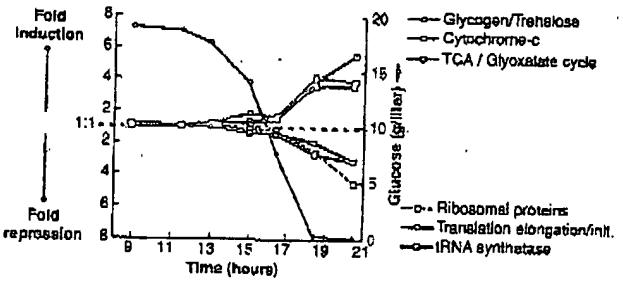

Fig. 4. Coordinated regulation of functionally related genes. The curves represent the average induction or repression ratios for all the genes in each indicated group. The total number of genes in each group was as follows: ribosomal proteins, 112; translation elongation and initiation factors, 25; tRNA synthetases (excluding mitochondial synthetases), 17; glycogen and trehalose synthesis and degradation, 15; cytochrome c oxidase and reductase proteins, 19; and TCA- and glyoxylate-cycle enzymes, 24.

Table 1. Genes induced by *YAP1* overexpression. This list includes all the genes for which mRNA levels increased by more than twofold upon *YAP1* overexpression in both of two duplicate experiments, and for which the average increase in mRNA level in the two experiments was greater than threefold (*50*). Positions of the canonical Yap1 binding sites upstream of the start codon, when present, and the average fold-increase in mRNA levels measured in the two experiments are indicated.

ORF	Distance of Yap1 site from ATG	Gene	Description	Fold-Increase
YNL331C			Putative aryl-alcohol reductase	12.9
YKL071W	162–222 (5 sites)		Similarity to bacterial csgA protein	10.4
YML007W		YAP1	Transcriptional activator involved in oxidative stress response	9.8
YFL056C	223, 242		Homology to aryl-alcohol dehydrogenases	9.0
YLL060C	98		Putative glutathione transferase	7.4
YOL165C	266		Putative aryl-alcohol dehydrogenase (NADP+)	7.0
YCR107W			Putative aryl-alcohol reductase	6.5
YML116W	409	ATR1	Aminotriazole and 4-nitroquinoline resistance protein	6.5
YBR008C	142, 167, 364		Homology to benomyl/methotrexate resistance protein	6.1
YCLX08C			Hypothetical protein	6.1
YJR155W			Putative aryl-alcohol dehydrogenase	6.0
YPL171C	148, 212	OYE3	NAPDH dehydrogenase (old yellow enzyme), isoform 3	5.8
YLR460C	167, 317		Homology to hypothetical proteins YCR102c and YNL134c	4.7
YKR076W	178		Homology to hypothetical protein YMR251w	4.5
YHR179W	327	OYE2	NAD(P)H oxidoreductase (old yellow enzyme), isoform 1	4.1
YML131W	507		Similarity to *A. thaliana* zeta-crystallin homolog	3.7
YOL126C		MDH2	Malate dehydrogenase	3.3

ing sites upstream of the others may reflect an ability of Yap1 to bind sites that differ from the canonical binding sites, perhaps in cooperation with other factors, or less likely, may represent an indirect effect of Yap1 overexpression, mediated by one or more intermediary factors. Yap1 sites were found only four times in the corresponding region of an arbitrary set of 30 genes that were not differentially regulated by Yap1.

Use of a DNA microarray to characterize the transcriptional consequences of mutations affecting the activity of regulatory molecules provides a simple and powerful approach to dissection and characterization of regulatory pathways and networks. This strategy also has an important practical application in drug screening. Mutations in specific genes encoding candidate drug targets can serve as surrogates for the ideal chemical inhibitor or modulator of their activity. DNA microarrays can be used to define the resulting signature pattern of alterations in gene expression, and then subsequently used in an assay to screen for compounds that reproduce the desired signature pattern.

DNA microarrays provide a simple and economical way to explore gene expression patterns on a genomic scale. The hurdles to extending this approach to any other organism are minor. The equipment required for fabricating and using DNA microarrays (9) consists of components that were chosen for their modest cost and simplicity. It was feasible for a small group to accomplish the amplification of more than 6000 genes in about 4 months and, once the amplified gene sequences were in hand, only 2 days were required to print a set of 110 microarrays of 6400 elements each. Probe preparation, hybridization, and fluorescent imaging are also simple procedures. Even conceptually simple experiments, as we described here, can yield vast amounts of information. The value of the information from each experiment of this kind will progressively increase as more is learned about the functions of each gene and as additional experiments define the global changes in gene expression in diverse other natural processes and genetic perturbations. Perhaps the greatest challenge now is to develop efficient methods for organizing, distributing, interpreting, and extracting insights from the large volumes of data these experiments will provide.

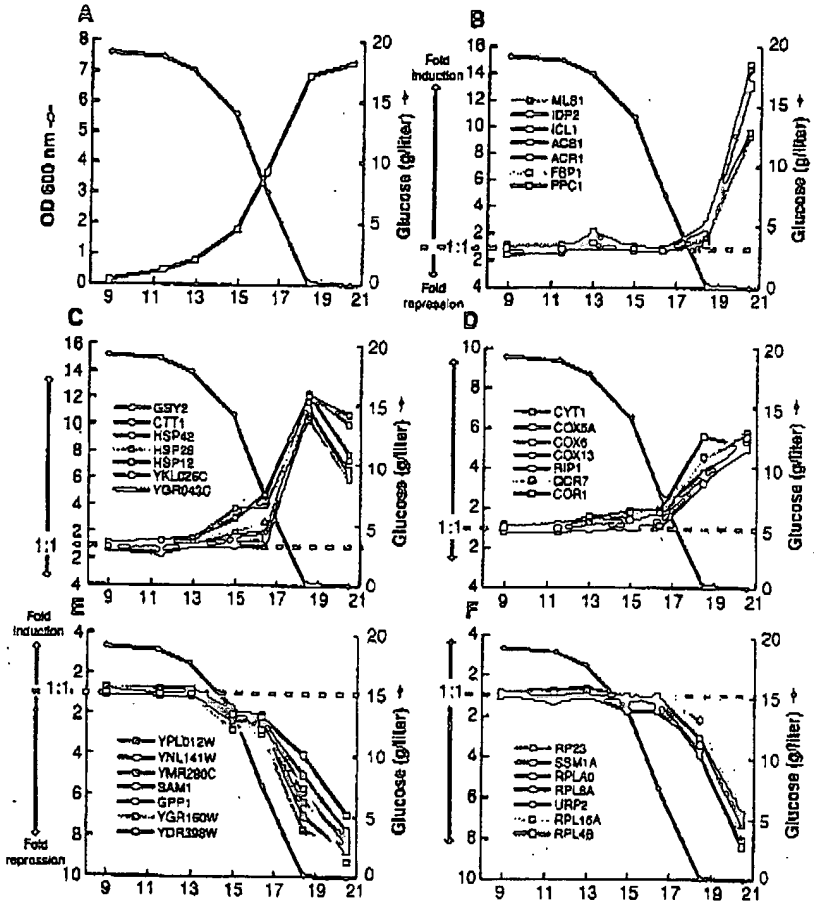

Fig. 5. Distinct temporal patterns of induction or repression help to group genes that share regulatory properties. (**A**) Temporal profile of the cell density, as measured by OD at 600 nm and glucose concentration in the media. (**B**) Seven genes exhibited a strong induction (greater than ninefold) only at the last timepoint (20.5 hours). With the exception of *IDP2*, each of these genes has a CSRE UAS. There were no additional genes observed to match this profile. (**C**) Seven members of a class of genes marked by early induction with a peak in mRNA levels at 18.5 hours. Each of these genes contain STRE motif repeats in their upstream promoter regions. (**D**) Cytochrome c oxidase and ubiquinol cytochrome c reductase genes. Marked by an induction coincident with the diauxic shift, each of these genes contains a consensus binding motif for the HAP2,3,4 protein complex. At least 17 genes shared a similar expression profile. (**E**) *SAM1*, *GPP1*, and several genes of unknown function are repressed before the diauxic shift, and continue to be repressed upon entry into stationary phase. (**F**) Ribosomal protein genes comprise a large class of genes that are repressed upon depletion of glucose. Each of the genes profiled here contains one or more RAP1-binding motifs upstream of its promoter. RAP1 is a transcriptional regulator of most ribosomal proteins.

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8. Primers for each known or predicted protein coding sequence were supplied by Research Genetics. PCR was performed with the protocol supplied by Research Genetics, using genomic DNA from yeast strain S288C as a template. Each PCR product was verified by agarose gel electrophoresis and was deemed correct if the lane contained a single band of appropriate mobility. Failures were marked as such in the database. The overall success rate for a single-pass amplification of 6116 ORFs was ~94.5%.
9. Glass slides (Gold Seal) were cleaned for 2 hours in a solution of 2 N NaOH and 70% ethanol. After rinsing in distilled water, the slides were then treated with a 1:5 dilution of poly-L-lysine adhesive solution (Sigma) for 1 hour, and then dried for 5 min at 40°C in a vacuum oven. DNA samples from 100-μl PCR reactions were purified by ethanol purification in 96-well microtiter plates. The resulting precipitates were resuspended in 3× standard saline citrate (SSC) and transferred to new plates for arraying. A custom-built arraying robot was used to print on a batch of 110 slides. Details of the design of the microarrayer are available at cmgm.stanford.edu/pbrown. After printing, the microarrays were rehydrated for 30 s in a humid chamber and then snap-dried for 2 s on a hot plate (100°C). The DNA was then ultraviolet (UV)-crosslinked to the surface by subjecting the slides to 60 mJ of energy (Stratagene Stratalinker). The rest of the poly-L-lysine surface was blocked by a 15-min incubation in a solution of 70 mM succinic anhydride dissolved in a solution consisting of 315 ml of 1-methyl-2-pyrrolidinone (Aldrich) and 35 ml of 1 M boric acid (pH 8.0). Directly after the blocking reac-

tion, the bound DNA was denatured by a 2-min incubation in distilled water at ~95°C. The slides were then transferred into a bath of 100% ethanol at room temperature, rinsed, and then spun dry in a clinical centrifuge. Slides were stored in a closed box at room temperature until used.

10. YPD medium (8 liters), in a 10-liter fermentation vessel, was inoculated with 2 ml of a fresh overnight culture of yeast strain DBY7286 (MATa, ura3, GAL2). The fermentor was maintained at 30°C with constant agitation and aeration. The glucose content of the media was measured with a UV test kit (Boehringer Mannheim, catalog number 716251). Cell density was measured by OD at 600-nm wavelength. Aliquots of culture were rapidly withdrawn from the fermentation vessel by peristaltic pump, spun down at room temperature, and then flash frozen with liquid nitrogen. Frozen cells were stored at −80°C.
11. Cy3-dUTP or Cy5-dUTP (Amersham) was incorporated during reverse transcription of 1.25 μg of polyadenylated [poly(A)+] RNA, primed by a dT(16) oligomer. This mixture was heated to 70°C for 10 min, and then transferred to ice. A premixed solution, consisting of 200 U Superscript II (Gibco), buffer, deoxyribonucleoside triphosphates, and fluorescent nucleotides, was added to the RNA. Nucleotides were used at these final concentrations: 500 μM for dATP, dCTP, and dGTP and 200 μM for dTTP. Cy3-dUTP and Cy5-dUTP were used at a final concentration of 100 μM. The reaction was then incubated at 42°C for 2 hours. Unincorporated fluorescent nucleotides were removed by first diluting the reaction mixture with of 470 μl of 10 mM tris-HCl (pH 8.0)/1 mM EDTA and then subsequently concentrating the mix to ~5 μl, using Centricon-30 microconcentrators (Amicon).
12. Purified, labeled cDNA was resuspended in 11 μl of 3.5× SSC containing 10 μg poly(dA) and 0.3 μl of 10% SDS. Before hybridization, the solution was boiled for 2 min and then allowed to cool to room temperature. The solution was applied to the microarray under a cover slip, and the slide was placed in a custom hybridization chamber which was subsequently incubated for ~8 to 12 hours in a water bath at 62°C. Before scanning, slides were washed in 2× SSC, 0.2% SDS for 5 min, and then 0.05× SSC for 1 min. Slides were dried before scanning by centrifugation at 500 rpm in a Beckman CS-6R centrifuge.
13. The complete data set is available on the Internet at cmgm.stanford.edu/pbrown/explore/index.html
14. For 95% of all the genes analyzed, the mRNA levels measured in cells harvested at the first and second interval after inoculation differed by a factor of less than 1.5. The correlation coefficient for the comparison between mRNA levels measured for each gene in these two different mRNA samples was 0.98. When duplicate mRNA preparations from the same cell sample were compared in the same way, the correlation coefficient between the expression levels measured for the two samples by comparative hybridization was 0.99.
15. The numbers and identities of known and putative genes, and their homologies to other genes, were gathered from the following public databases: *Saccharomyces* Genome Database (genome-www.stanford.edu), Yeast Protein Database (quest7.proteome.com), and Munich Information Centre for Protein Sequences (speedy.mips.biochem.mpg.de/mips/yeast/index.html).
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36. For example, we observed large inductions of the genes coding for *PCK1*, *FBP1* [Z. Yin *et al.*, *Mol. Microbiol.* **20**, 751 (1996)], the central glyoxylate cycle gene *ICL1* [A. Scholer and H. J. Schuller, *Curr. Genet.* **23**, 375 (1993)], and the "aerobic" isoform of acetyl-CoA synthase, *ACS1* [M. A. van den Berg *et al.*, *J. Biol. Chem.* **271**, 28953 (1996)], with concomitant down-regulation of the glycolytic-specific genes *PYK1* and *PFK2* [P. A. Moore *et al.*, *Mol. Cell. Biol.* **11**, 5330 (1991)]. Other genes not directly involved in carbon metabolism but known to be induced upon nutrient limitation include genes encoding cytosolic catalase T *CTT1* [P. H. Bissinger *et al.*, *ibid.* **9**, 1309 (1989)] and several genes encoding small heat-shock proteins, such as *HSP12*, *HSP26*, and *HSP42* [I. Farkas *et al.*, *J. Biol. Chem.* **266**, 15602 (1991); U. M. Praekelt and P. A. Meacock, *Mol. Gen. Genet.* **223**, 97 (1990); D. Wotton *et al.*, *J. Biol. Chem.* **271**, 2717 (1996)].
37. The levels of induction we measured for genes that were expressed at very low levels in the uninduced state (notably, *FBP1* and *PCK1*) were generally lower than those previously reported. This discrepancy was likely due to the conservative background subtraction method we used, which generally resulted in overestimation of very low expression levels (*46*).
38. Cross-hybridization of highly related sequences can also occasionally obscure changes in gene expression, an important concern where members of gene families are functionally specialized and differentially regulated. The major alcohol dehydrogenase genes, *ADH1* and *ADH2*, share 88% nucleotide identity. Reciprocal regulation of these genes is an important feature of the diauxic shift, but was not observed in this experiment, presumably because of cross-hybridization of the fluorescent cDNAs representing these two genes. Nevertheless, we were able to detect differential expression of closely related isoforms of other enzymes, such as *HXK1/HXK2* (77% identical) [P. Herrero *et al.*, *Yeast* **11**, 137 (1995)], *MLS1/DAL7* (73% identical) (*20*), and *PGM1/PGM2* (72% identical) [D. Oh, J. E. Hopper, *Mol. Cell. Biol.* **10**, 1415 (1990)], in accord with previous studies. Use in the microarray of deliberately selected DNA sequences corresponding to the most divergent segments of homologous genes, in lieu of the complete gene sequences, should relieve this problem in many cases.
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50. In addition to the 17 genes shown in Table 1, three additional genes were induced by an average of more than threefold in the duplicate experiments, but in one of the two experiments, the induction was less than twofold (range 1.6- to 1.9-fold).
51. We thank H. Bennett, P. Spellman, J. Ravetto, M. Eisen, R. Pillai, B. Dunn, T. Ferea, and other members of the Brown lab for their assistance and helpful advice. We also thank S. Friend, D. Botstein, S. Smith, J. Hudson, and D. Dolginow for advice, support, and encouragement; K. Struhl and S. Chatterjee for the Tup1 deletion strain; L. Fernandes for helpful advice on Yap1; and S. Klapholz and the reviewers for many helpful comments on the manuscript. Supported by a grant from the National Human Genome Research Institute (NHGRI) (HG00450), and by the Howard Hughes Medical Institute (HHMI). J.D.R. was supported by the HHMI and the NHGRI. V.R. was supported in part by an Institutional Training Grant in Genome Science (T32 HG00044) from the NHGRI. P.O.B. is an associate investigator of the HHMI.

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